Elucidating the Regulation and Effectors of the Breast Cancer Oncogene, IKKepsilon

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Abstract

The IkappaB kinase epsilon (IKKepsilon, IKKi, *IKBKE*) is both a regulator of innate immunity and a breast cancer oncogene that is amplified and overexpressed in ~30% of breast cancers. IKKepsilon promotes malignant transformation through the activation of NF-kappaB signaling. In addition, breast cancers that harbor amplifications in the *IKBKE* gene are dependent on IKKepsilon protein expression for survival. IKKepsilon has been characterized as a non-canonical inhibitor of kappaB kinase (IKK) that activates both the interferon response pathway and NF-kappaB signaling in innate immunity. In this dissertation, I explore both the regulation and effectors of the IKKepsilon kinase in the context of malignant transformation. I found that IKKepsilon is modified and regulated by K63-linked polyubiquitination, a proteasome- and degradation-independent form of ubiquitination, at Lysine 30 and Lysine 401. This modification is essential for IKKepsilon-induced kinase function and IKKepsilon-mediated NF-kappaB activation and malignant transformation. Furthermore, I identified TRAF2 as the K63 ubiquitin E3 ligase that associates with and modifies IKKepsilon. I also found that TBK1, a close family member of IKKepsilon, is also regulated by K63-linked ubiquitination. In collaborative work, we used an unbiased positional scanning peptide library screen to identify two novel downstream targets of IKKepsilon phosphorylation in the

context of cancer. Specifically, we found IKKepsilon phosphorylates the tumor suppressor CYLD at Serine 418. CYLD phosphorylation at Ser418 downregulates its deubiquitinase activity and is necessary for IKKepsilon-driven transformation. IKKepsilon also phosphorylates TRAF2 at Serine 11. This activity promotes K63-linked TRAF2 ubiquitination, NF-kappaB activation and is also essential for IKKepsilon-transformation. In addition, breast cancer cells that depend on IKKepsilon expression for survival are also dependent on TRAF2. Together, these observations define an oncogenic network that promotes NF-kappaB-mediated cell transformation through the K63-linked ubiquitination of IKKepsilon and subsequent phosphorylation of two novel substrates, TRAF2 and CYLD.

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CHAPTER ONE

INTRODUCTION

Nuclear factor κB (NF-κB) is a central transcriptional regulator of many important biological processes including innate immunity, inflammation, cell proliferation and apoptosis [1-4]. These NF-κB-driven processes have also been defined to be important hallmarks of cancer pathogenesis [5, 6]. As a result, aberrant NF-κB signaling has been linked as the mechanistic basis for many cancers either due to aberrant NF-κB activation in the tumor microenvironment, which leads to inflammation, or dysregulation of specific NF-κB pathway proteins within the tumor itself.

The NF-κBs are a family of proteins that were initially characterized due to their interaction with the immunoglobulin light-chain enhancer in B cells [7]. The NF-κB family of transcription factors is composed of five proteins: RelA(p65), RelB, c-Rel, p105/p50(NFκB1) and p100/p52(NFκB2) [8]. These proteins share a conserved Rel homology domain (RHD), a ~300 amino acid domain that conveys DNA binding specificity. A subset of the NF-κB proteins – RelA, RelB and c-Rel – also share a C-terminal transactivation domain [9]. p105(NFκB1) and p100(NFκB2) are synthesized as large precursors that undergo processing by degradation to become their smaller, active forms: p50 and p52 respectively [10]. The processing of p105 and p100 is an ubiquitin-proteasome pathway mediated process that results in the specific degradation of the C-terminal ankyrin repeat (AR) containing portion of the precursor. In their active forms, NF-κB proteins form homo- and hetero-dimers to act as DNA-binding transcription factors and promote the activation of downstream targets [11, 12].

The NF-κB proteins are bound and kept inactive in the cytoplasm by a family of NF-κB-binding proteins known as the inhibitors of NF-κB (IκBs). The activation of the NF-κB pathway by a variety of proinflammatory stimuli and cytokine receptors results in

the ubiquitin-mediated degradation of the IκB proteins and subsequent release and translocation of the NF-κB dimers into the nucleus [12]. The ubiquitination of the IκB proteins is triggered by a phosphorylation event that is mediated by a collection of inhibitor of κB kinases (IKKs). The IKK family of proteins include the catalytically active IKKα (IKK1), IKKβ (IKK2), IKKε (IKK-i), TBK1 (NAK) and the catalytically inactive IKKγ (NEMO). It is the differential regulation of these IKK proteins that allows for the fine-tuned control of the NF-κB response pathway to various stimuli.

Various types of ubiquitination also play important and differing roles throughout the process of NF-κB signaling [13]. Ubiquitin is a 76 amino acid covalent protein modification that is attached to substrates through a three-enzyme cascade. First, the ubiquitin-activating enzyme (E1) uses ATP to forms a thiol-ester bond between the C-terminal glycine of ubiquitin and the active-site cysteine of the E1. This activated ubiquitin is then transferred to the cysteine of an ubiquitin-conjugating enzyme (E2), resulting in the formation of an E2-ubiquitin bond. In the last step of the process, the ubiquitin ligase (E3) enzyme transfers the charged ubiquitin from the E2 to the substrate by forming an isopeptide bond between the C-terminal glycine of ubiquitin and the ε-amino group of a lysine residue on the substrate [14]. Ubiquitin itself contains seven internal lysines (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63) and polyubiquitin chain formation can occur from any one of these seven lysines [15, 16]. In addition, recent work has also characterized Met1 linear polyubiquitination, which is the formation of a head-to-tail polyubiquitin chain [17, 18]. The linkage type of the polyubiquitin chain can result in very different fates for the ubiquitinated substrate. To date, only Lys11(K11), Lys48(K48), Lys63(K63) and Met1 polyubiquitination has been observed at

a significant level in eukaryotic cells, though polyubiquitination of all seven linkage types is possible *in vivo* [19, 20]. K48-linked polyubiquitination is the most well-studied and well-known type of ubiquitin linkage and results in the canonical proteasome-mediated degradation of the substrate [21, 22]. K11-linked ubiquitination occurs at a very low frequency in mammalian cells and seems to mostly recapitulate the effects of K48-linked ubiquitination [16]. K63- and Met1- linked polyubiquitination are thought to positively regulate their protein targets by recruiting other ubiquitin-binding proteins and further activate downstream signaling [23, 24]. K48-, K63- and Met1-linked polyubiquitination have all been shown to play essential roles in both the positive and negative regulation of the NF-κB pathway [18, 25-28].

CANONICAL AND NON-CANONICAL NF-κB SIGNALING

The NF-κB signaling pathways can be broadly classified as canonical and non-canonical depending upon whether a NEMO-dependent IKK signal cascade is involved (canonical). In addition, canonical NF-κB signaling generally involves the release of pre-existing NF-κB dimers from IκBs while non-canonical NF-κB signaling results in the novel synthesis of p100 and RelB (p52:RelB) dimers and is IκB independent [9, 29]. Both pathways are graphically illustrated in Figure 1.1.

CANONICAL NF-κB SIGNALING

The canonical pathway is activated in response to cytokine receptors including TNF-α receptor (TNFR), IL-1 receptor (IL-1R), Toll-like receptors (TLRs) and antigen receptors [13, 24]. Upon receptor-ligand binding, a signaling cascade is initiated that

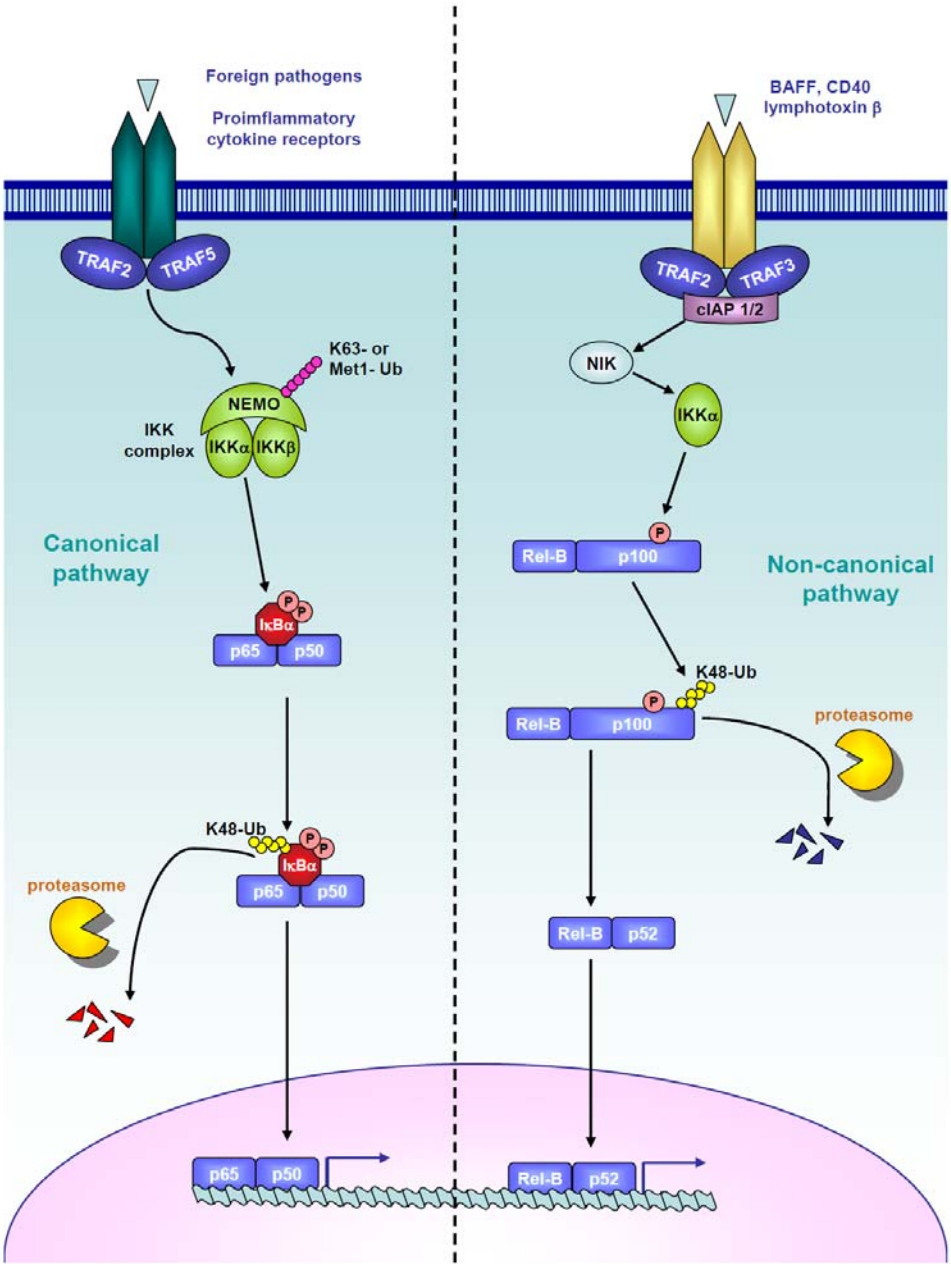

Figure 1.1. Canonical and non-canonical NF-κB activation. In the canonical NF-κB pathway (left), stimulation of proimflammatory receptors leads to eventual activation of the IKK complex, which in turn phosphorylates IκB proteins and targets them for K48-linked polyubiquitination and proteasome-mediated degradation. This frees the p50/p65 NF-κB dimer to enter the nucleus and activate gene transcription. In the non-canonical NF-κB pathway (right), stimulation of a subset of receptors, including the BAFF and CD40 receptors, results in the eventual stabilization of the NIK kinase which allows for IKKα activation. IKKα phosphorylates p100, resulting in its K48-linked ubiquitination and proteasome-mediated processing to p52. The free p52/Rel-B dimer then translocates into the nucleus to activate gene transcription.

ultimately converges upon the activation of the IKK complex. The IKK complex is composed of three main components: IKKα, IKKβ, and NEMO [30]. Activation of the IKK complex involves the recruitment of several ubiquitination enzymes and ultimately results in the activation of IKKβ by phosphorylation at serines 177 and 181 [31].

Currently, there are three known pathways for IKKβ activation that are differentially utilized depending on the receptor activation pathway. TNFR activation results in the recruitment of the E2 ubiquitin enzyme, UbcH5, and the E3 ubiquitin ligase, cIAP1. The recruitment of this E2/E3 complex results in the polyubiquitination of RIP1 by various ubiquitin chain linkages [32, 33]. This polyubiquitination recruits the TAB/TAK1 and IKK complexes together and allows for TAK1 to phosphorylate and activate IKKβ [34]. Alternatively, the bringing together of several IKK complexes can also lead to TAK1-independent *trans*-phosphorylation and activation of IKKβ. Another pathway of IKKβ activation has been recently described to involve the formation of Met1-linked linear polyubiquitination. In TNFR signaling, TRADD, TRAF2 and cIAP1/2 recruit the linear ubiquitin ligase complex, LUBAC [27]. LUBAC catalyzes the linear Met1-linked ubiquitination of NEMO, resulting in *trans*-autophosphorylation and activation of IKKβ [28]. Finally, in the third pathway of activation usually seen in IL-1R and TLR signaling, the E2/E3 complex of Ubc13/TRAF6 is recruited to form K63-linked ubiquitin chains on IRAK1 [35]. This allows for TAK1 and IKKβ recruitment to IRAK1, leading to the phosphorylation and activation of IKKβ by TAK1.

Upon IKKβ activation, the IKK complex phosphorylates IκBα at serines 32 and 36, which leads to the subsequent K48-linked polyubiquitination of lysines 21 and 22. This combination of modifications results in the degradation of IκBα via the proteasome [36].

In canonical NF-κB signaling, the main NF-κB dimers are formed between the transactivating domain-containing RelA and c-Rel together with the transactivating domain-lacking p50 [9]. Upon degradation of IκBα, the p50/p65 dimers are free to translocate into the nucleus where they can influence the gene transcription of downstream targets. Interestingly, one of the genes that is upregulated in this pathway is IκBα itself. This allows for the pathway to be rapidly shutoff in the absence of persistent stimulation. The canonical NF-κB pathway is also negatively regulated by A20, which is simultaneously a K48-linkage specific E3 ligase and a K63-linkage specific deubiquitinase (DUB) [37].

NON-CANONICAL NF-κB SIGNALING

The most well-studied non-canonical NF-κB pathway is activated in response to a subset of the TNF ligands including, BAFF, CD40 and lymphotoxin-β [38]. This pathway is NEMO-independent and is ultimately regulated by an upstream signaling complex that involves cIAP1/2, TRAF2, TRAF3 and NF-κB-inducing kinase (NIK) [39]. The NIK protein is continuously synthesized but is constitutively degraded through K48-linked ubiquitination. In the non-canonical NF-κB pathway, receptor stimulation initiates TRAF2 activation, which results in the recruitment and K63-linked ubiquitination of cIAP1/2. The activated cIAP1/2 and TRAF2 is then recruited into complex by TRAF3. This results in TRAF3, itself, undergoing K48-linked ubiquitination by cIAP1/2 and subsequent degradation [40]. Without TRAF3, NIK is no longer degraded, allowing for the accumulation of NIK protein [41, 42]. NIK then phosphorylates and activates IKKα, which promotes the IKKα-mediated phosphorylation of p100 [39, 43, 44].

Phosphorylation of p100 leads to the proteasome-mediated processing of p100 to the active p52, which in complex with RelB is liberated to translocate into the nucleus and activate downstream NF-κB effectors.

IKKε AND TBK1

Aside from IKKα and IKKβ, there exist two other catalytically active IKK proteins: IκB kinase ε (IKKε, IKK-i, *IKBKE*) and TANK-binding kinase 1 (TBK1, T2K, *TBK1*). Two groups independently identified IKKε at the same time. The first utilized a suppression subtractive hybridization screen to identify IKKε as an LPS-inducible gene in mouse macrophages [45]. The second found IKKε to be part of a novel PMA-inducible IκB kinase complex that is capable of NF-κB activation [46]. Around the same time, TBK1 was also identified as a binding partner for TRAF-associated NF-κB activator (TANK) that, together with TRAF2, activates NF-κB in a kinase-dependent manner [47].

IKKε and TBK1 share 27% identity to the canonical IKKα and IKKβ proteins [39, 45, 47]. However, they share 49% identity and 65% similarity to one another [48]. Both proteins are comprised of an N-terminal kinase domain, an ubiquitin-like domain, an ubiquitin-binding domain, and C-terminal leucine-zipper (LZ) and helix-loop-helix (HLH) regions [48]. Both kinases also contain a coiled-coil multimerization domain, which allows them to form homo- and hetero-dimers with one another. IKKε is specifically expressed in a subset of tissues that are important for immune function: the thymus, spleen, peripheral blood leukocytes, pancreas and placenta [45]. TBK1, on the other hand, is ubiquitously expressed throughout all organ systems [47].

IKKε AND TBK1 IN INTERFERON SIGNALING

TBK1 and IKKε play an essential role in the interferon response pathway to viral challenge [49, 50]. The interferon response pathway is normally triggered by recognition of viral dsRNA by TLRs. TLR activation results in the recruitment of a complex containing IKKε, TBK1, TANK and TRAF3 [51]. Activated IKKε and TBK1 further phosphorylate a set of interferon regulatory factors (IRFs), IRF3 and IRF7 [52-54]. Phosphorylation of the IRFs allows them to dimerize and translocate into the nucleus where they can promote the transcription of proinflammatory and antiviral genes including interferon-α and -β (IFNα and IFNβ) [55, 56].

Aside from TLR-induced signaling, IKKε and TBK1 can also be activated in a TLR-independent manner. The recognition of viral dsRNA and dsDNA by DNA- and RNA-sensing proteins such as RIG-I, MDA-5, and DAI can lead to IKKε-mediated NF-κB activation [57-62]. In addition, IFNβ itself can also stimulate IKKε binding to a large interferon activating complex known as ISGF3, resulting in the IKKε-dependent activation of a subset of interferon response genes [63]. IKKε is also shown to play a critical role in the balance between activation of the type I and type II (IFN-I and IFN-II) interferon signaling pathways through ISFGF3[64]. ISGF3 is a multimeric transcriptional activation complex that is composed of STAT1, STAT2, and IFN regulatory factor 9. IFN-II promotes the homodimerization of STAT1, resulting in the formation of the gamma-activated factor (GAF) complex. ISGF3 and GAF differentially control the transcription of IFN-I and IFN-II response genes to activate distinct sets of antiviral genes. IKKε has been shown to phosphorylate STAT1, which prevents its dimerization

and subsequent GAF formation. This allows IKKε to control the balance of IFN-I and IFN-II signaling by differential activation of ISGF3 and GAF [64].

TBK1 and IKKε each bind several different proteins. Like the canonical IKKs, the kinase activity of TBK1 and IKKε is increased when they are a part of a larger high-molecular-weight complex. TBK1 and IKKε have been shown to complex with a variety of scaffolding proteins and other enzymes, including NAP1, SINTBAD, MAVS, TANK, TRAF2, TRAF3 and TRADD to mediate interferon activation [65-69]. The composition of the protein complex differs depending on the stimulus, indicating that IKKε and TBK1 can be modulated and activated differentially depending on protein complex composition and formation [48].

IKKε AND TBK1 IN NF-κB SIGNALING

TBK1 and IKKε are classified as non-canonical NF-κB activators due to their ability to catalyze the phosphorylation of IκBα in a NEMO-independent manner. In the canonical pathway, IκBα requires two phosphorylation events at Ser36 and Ser32 to be targeted for degradation. IKKε preferentially phosphorylates IκBα Ser36 [45, 46]. On the other hand, TBK1 preferentially phosphorylates IκBα Ser32 [47, 70]. Interestingly, overexpression of IKKε alone results in the reduction of IκBα protein levels, suggesting that perhaps Ser36 phosphorylation alone is sufficient to induce IκBα degradation. Or, an alternative possibility is that IKKε and TBK1 are able to recruit a canonical IKK protein to mediate the phosphorylation and degradation of IκBα [71, 72].

TBK1 and IKKε are also capable of directly phosphorylating some of the NF-κB transcription factors. IKKε and TBK1 have been shown to work with the canonical IKKα/β kinases to phosphorylates RelA(p65) at Ser536 at a basal stimulus-free level [73, 74]. This basal RelA phosphorylation is believed to be important for the recruitment of basal transcriptional machinery to the interleukin-8 promoter. Another NF-κB transcription factor, cRel, has also been shown to be a kinase target of TBK1 and IKKε. This phosphorylation of cRel is sufficient to promote nuclear translocation, but does not further activate downstream NF-κB activity [75].

DISTINCT ROLES OF IKKε AND TBK1

Despite their similarity, TBK1 and IKKε also each have a distinct set of non-overlapping targets. TBK1 is known to phosphorylate and activate IKKβ at Ser177 and 181, resulting in the activation of the canonical NF-κB pathway [70]. Also, in the context of innate immunity signaling, TBK1 phosphorylates Sec5 and DDX3X to induce the interferon response [76, 77]. On the other hand, IKKε has been shown to uniquely phosphorylate RelA at Ser468 to facilitate NF-κB signaling, and also STAT1 in the context of ISGF3-dependent interferon activation [63, 78].

To further support the idea that TBK1 and IKKε play distinct though related roles, generation of the *Tbk1*$^{-/-}$ and *Ikbke*$^{-/-}$ genetic knockout mice showed that these two mouse models also have distinct phenotypes. *Tbk1* deficient mice are embryonic lethal and die after E14.5 due to liver degeneration [79, 80]. This phenotype is also prevalent in NEMO, IKKβ and RelA deficient mice, indicating that TBK1 may play a role in the same pathway as these canonical NF-κB pathway effectors. *Ikbke* deficient mice, on the other

hand, are viable. They are unable to effectively mount the interferon response to viral infection, but are otherwise normal when unperturbed [80]. Interestingly, both the *Ikbke* and *Tbk1* deficient mice are relatively normal in the context of NF-κB induction and signaling. This genetic evidence indicates that, although TBK1 and IKKε are capable of NF-κB induction, their roles are non-essential to the proper activation of this pathway. On the other hand, TBK1 and IKKε are critical for the proper stimulation of the interferon response pathway.

NF-κB ACTIVATION IN CANCER

Aside from its normal role in the activation of the proinflammatory response to pathogenic challenge, the NF-κB pathway has also been implicated as the mechanistic link between inflammation and cancer [81-83].

Inhibition of various canonical NF-κB pathway effectors has been shown to decrease disease progression in several inflammation-associated cancer models. In the colitis-associated cancer (CAC) mouse model [84], the canonical NF-κB kinase, IKKβ, was shown to play an essential role for disease progression in two distinct cell types [85]. Deletion of IKKβ in the malignant enterocytes activated the anti-apoptotic genes to suppress the elimination of these cells. In addition, deletion of IKKβ in the myeloid microenvironment cells promoted the secretion of various cytokines that supported the growth of the malignant enterocytes. The overall result was that IKKβ deletion resulted in a ~80% decrease in tumor incidence in the CAC mouse model. In an *Mdr2*-knockout inflammatory hepatocellular carcinoma mouse model [86], the introduction of the NF-κB

canonical pathway super repressor into hepatocytes inhibited tumor progression and induced apoptosis [87]. Similar to the CAC model, the microenvironment endothelial cells secrete TNF-α to support the growth of these malignant hepatocytes. As a result, blocking TNF-α signaling was sufficient to inhibit NF-κB activation in the malignant hepatocytes. In both of these examples, aberrant NF-κB signaling plays both a cell-autonomous and a cell-non-autonomous role in the promotion of the disease phenotype. Within the tumor cell itself, NF-κB activation promotes an anti-apoptotic phenotype; while in the microenvironment, NF-κB activation promotes the secretion of extracellular factors to promote cell survival and growth of the tumor cells.

Aberrant activation of NF-κB pathway effectors within the tumor cells was first observed in blood cancers. For example, *NFKB2* is commonly involved in translocations in hematological malignancies [88, 89]. Upstream mutations that lead to NF-κB activation are common in MALT lymphomas [90]. Activating mutations in Carma-1 and MyD88, which lead to the aberrant activation of NF-κB, have been detected in B cell lymphomas [91, 92]. In multiple myeoloma, many NF-κB pathway mutations have been observed including mutations in the IKK proteins (*IKBKB*), TRAF adaptor proteins (*TRAF2, TRAF3)*, and NF-κB transcription factors (*NFKB2)*, as well as mutations that lead to the constitutive activation of NIK [42, 93-96].

However, aberrant NF-κB pathway activation is most commonly observed in solid tumors deriving from epithelial cells. Mutations in *IKKA* and *IKBKB* have been found through genomic sequencing of breast and prostate cancers [97, 98]. Aberrant IKKα signaling has been shown to promote the self-renewal of breast cancer progenitors, play a role in the tumor-promoting effects of progesterone, as well as promote the

metastatic spread of breast cancer [99-102]. In addition, breast cancer cell lines and transformed mammary epithelial cells have been shown to have high levels of nuclear NF-κB/Rel dimers as compared to normal breast tissue, implicating a role for NF-κB activation in breast cancer. A large scale study that interrogated copy number alterations in 3131 human cancer tissues also identified significant somatic copy-number alterations of many NF-κB effectors including *TRAF6, IKBKB, IKBKG, IRAK1* and *RIP1* in epithelial cancers [103].

IKKε AND TBK1 IN CANCER

In addition to canonical NF-κB effectors, other recent studies have suggested that the non-canonical IKKs, IKKε and TBK1 may also play a role in driving NF-κB activation in cancer.

IKKε IN CANCER

IKKε was first implicated in cancer when it was identified to be overexpressed in human breast cancer samples, breast cancer cell lines, and DMBA-induced mouse mammary tumors [71]. In this study, IKKε was overexpressed in four out of six primary human breast cancer samples. In breast cancer cell lines, IKKε was expressed at higher levels in MDA-MB 231, Hs578T, MDA-MB 468 and T47D cells as compared to untransformed MCF10F breast epithelial cells. In addition, six out of seven DMBA-induced mouse mammary tumors exhibited higher levels of IKKε expression as compared to the normal mouse mammary tissue. This study also showed that IKKε

expression was increased in the mammary tumors derived from two mammary-specific transgenic mouse models, MMTV-CK2α and MMTV-*c-rel*. Ectopic expression of CK2 in NIH 3T3 mouse fibroblasts, MCF10F and HEK293T cells induced the expression of IKKε and chemical inhibition of CK2 in breast cancer cell lines that overexpress IKKε showed a decrease in IKKε expression. These observations suggested that CK2 may play a role in the regulation of IKKε [71]. Another study found IKKε protein levels to be elevated in a panel of prostate and breast cancer cell lines [104]. In this study, IKKε was shown to catalyze the basal phosphorylation of RelA at Ser536. The phosphorylation was shown to be constitutive, non cytokine-induced, and was proposed to play a role in the transactivation of NF-κB [104]. These two studies implicated a role for IKKε in breast cancer.

In 2007, *IKBKE*, was shown to be a breast cancer oncogene through the integration of three functional genomics approaches [72]. In a gain-of-function approach, Boehm *et al.* utilized a human epithelial cell transformation model in which transformation was driven by a combination of activated MEK and activated AKT. Through a comprehensive kinase screen, *IKBKE* was identified as capable of replacing AKT in this model to induce malignant cell transformation as assessed by soft agar colony formation and tumor formation in immunocompromised mice. In a second approach, Boehm *et al.* utilized a loss-of-function screen to identify specific genetic vulnerabilities of breast cancer cell lines and again found *IKBKE* to be an essential gene for breast cancer survival. Finally, in the third approach, *IKBKE* was found to be amplified in 8 out of 49 (16.3%) of breast cancer cell lines and ~30% of primary breast cancer tumors. Taken together, the intersection of these three approaches identifies

IKBKE as an essential breast cancer oncogene that is significantly amplified and overexpressed in breast cancer cell lines and tumors. In this study, IKKε-transformed human mammary epithelial exhibited increased expression of NF-κB response genes and, importantly, IKKε-mediated malignant cell transformation was blocked by the expression of the NF-κB super repressor. In contrast, suppression of IRF3 and IRF7 by shRNA expression did not affect the ability of IKKε to mediate transformation. These data taken together suggest that the mechanism of IKKε-mediated transformation is through its role as an NF-κB activator and is independent of its role in the interferon response pathway [72].

Although *IKBKE* copy number gain does not correlate with estrogen receptor (ER) or Her2/Neu status [72], one study has shown that IKKε phosphorylates ER-α and contributes to tamoxifen resistance in breast cancer [105]. Guo *et al.* showed that IKKε phosphorylates ER-α at Ser167 both *in vitro* and *in vivo* and that this phosphorylation results in the transactivation of ERα. As a result of this phosphorylation, cyclin D1, a major ERα target, is upregulated. In addition, this study showed that high expression of IKKε induces tamoxifen-resistance, and that suppression of IKKε in these cells resulted in increased tamoxifen-sensitivity [105].

In addition to breast cancer, IKKε has also been shown to have a prognostic role in several other cancer types including ovarian cancer, prostate cancer, glioma, pancreatic ductal adenocarcinoma, clear cell renal cell carcinoma, and non-small cell lung cancer [106-111]. In prostate cancer, a recent study surveyed IKKε expression via immunostaining in paraffin-embedded prostate tissue microarrays. The investigators

surveyed both normal tissue (47 normal tissues, 53 non-malignant tumor-adjacent tissues) and prostate cancer samples at various stages of disease (28 prostatic intraepithelial neoplasia (PIN) lesions, 62 hormone-sensitive and 31 castrate-resistant tumors). This study showed IKKε expression to be upregulated in malignant prostate cancer samples as compared to normal tissue, with the highest IKKε expression found in the castrate-resistant tumor samples [106]. In ovarian cancer, a study showed that IKKε is overexpressed in 63 of 95 ovarian cancer samples and that high levels of IKKε were associated with poor disease prognosis in these samples [107]. In glioma, high IKKε was found in 38 out of 71 paraffin-embedded glioma samples [108]. This study also showed that IKKε conveyed resistance to apoptotis to glioma cells that were tranduced with IKKε expression, implicating an oncogenic role for IKKε in the context of glioma [108]. In clear cell renal cell carcinoma, expression profiling of 93 tumor samples revealed that high IKKε expression levels was correlated with a 5.3fold increase in patient mortality [109]. In pancreatic ductal adenocarcinoma, high IKKε expression was observed in 50 out of 78 tumor samples [110]. Finally, in non-small cell lung cancer, IKKε expression was shown to be induced by STAT3 activation in the context of tobacco and nicotine exposure [111]. All of these studies taken together implicate IKKε as not only an important player in breast cancer, but possibly a useful prognostic tool in other cancers as well.

TBK1 IN CANCER

TBK1 was identified to be highly expressed in lung, breast and colon cancers. In addition, a TBK1 mutation, P675L was identified in lung adenocarcinoma [112-114].

TBK1 and IRF3 were identified to promote angiogenesis through a high throughput gain of function screen. HEK293T cells were transfected with a comprehensive genome-encompassing cDNA library, the conditioned media from these transfected cells was collected and fed to human umbilical vein endothelial cells (HUVECs) to determine which genes could trigger the production of proangiogenic secreted factors [112]. TBK1, TRIF, and IRF3 all scored positively in this screen as triggers. Further analysis revealed that TBK1 stimulated the production of several different secreted factors including the endothelial growth factors RANTES, IL-8. This pro-angiogenic fuction of TBK1 expression began to link TBK1 as a possible player in cancer progression [112].

A later study mechanistically linked TBK1 to tumorigenesis by showing its association with RalB [76]. RalB is a monomeric RalGTPase, a part of the RasGTPase superfamily, that has been implicated as an essential component of Ras-induced oncogenic transformation. RalB is required to suppress the apoptotic checkpoint activation. RalB recruits the binding of the exocyst complex, a large super complex composed of eight core secretory proteins. One of these proteins, Sec5, was shown to be essential for RalB mediated apoptotic control. In a study to understand the mechanism of RalB/Sec5 action, Chien *et al.* identified TBK1 to be actively recruited to the RalB/Sec5 complex. Furthermore, depletion of TBK1 protein by siRNA mimicked the effects of RalB and Sec5 depletion, resulting in TBK1 deficient cells undergoing an asynchronous apoptotic program. Importantly, TBK1 deficient MEFs were unable to undergo Ras-induced oncogenic transformation as compared to their wildtype counterparts. Instead, transduction of TBK1 deficient MEFs with oncogenic K-RasG12V resulted in apoptosis [76].

TBK1 has also been implicated in RAS signaling as a protein whose suppression resulted in synthetic lethality in oncogenic *KRAS*-dependent lung cancer cell lines [114]. Barbie *et al.* found that cell lines that are dependent on mutant *KRAS* expression are also dependent on TBK1. Expression profile analyses were performed on patient-derived tumors from 128 lung adenocarcinomas. Of 19 mutant *KRAS* tumors, 14 showed an enrichment in the NF-κB activation signature. Expression of the NF-κB super repressor induced the selective apoptosis of *KRAS* mutant cell lines. These data provides evidence that TBK1 and NF-κB activation is a co-dependency for *KRAS*-dependent lung cancers [114].

Recent work by two independent groups has identified AKT as a downstream phosphorylation target of both IKKε and TBK1 in the context of cancer [115, 116]. Since IKKε was originally identified as a breast cancer oncogene by replacing AKT in a gain-of-function screen [72], it would follow that AKT is likely a downstream target of IKKε signaling. Overexpression of wildtype but not kinase-dead IKKε or TBK1 resulted in an increase in AKT phosphorylation at Ser473 and Thr308 as well an increase in phosphorylation of the AKT substrates FoxO1, TSC2, $p70^{S6K}$ and GSK-3. IKKε and TBK1 were capable of phosphorylating AKT *in vitro* in a dose-dependent manner. Both groups also showed that IKKε/TBK1-mediated phosphorylation of AKT was independent of mTORC2. However, whether AKT phosphorylation is dependent on PI3K signaling remains controversial. One group showed that the treatment IKKε-expressing *Ikbke*$^{-/-}$ MEFs by the PI3K inhibitors LY294002 and Wortmanin did not affect IKKε-induced AKT phosphorylation [107]. On the other hand, the other group performed a similar experiment and showed that LY294002 and Wortmanin treatment significantly decreased

the ability of IKKε to phosphorylate AKT [116]. Thus, the overlap of IKKε and PI3K signaling remains unclear.

ELUCIDATING THE REGULATION AND EFFECTORS OF IKKε IN CANCER

Although the mechanism of IKKε signaling in the context of innate immunity and proinflammatory NF-κB signaling has been studied, the pathway and regulation of IKKε signaling in the context cancer remains relatively unknown.

Both degradation-dependent K48-linked and degradation-independent K63- and Met1-linked ubiquitination have been shown to play a critical role in the regulation of various members of the canonical NF-κB pathway [13]. In my dissertation work, I explored the regulation of IKKε by ubiquitination and determined that IKKε undergoes K63-linked degradation-independent ubiquitination in the context of breast cancer (Chapter 2).

IKKε has been shown to have several substrates in the context of innate immunity and NF-κB activation. However, the downstream effectors of IKKε signaling in the context of breast cancer have not been well-studied. In collaborative work with Rhine Shen and the Cantley lab, we undertook an unbiased positional peptide screen in order to identify novel IKKε kinase targets. Through this approach, we identified and characterized two novel and important substrates for IKKε phosphorylation in the context of breast cancer, CYLD (Chapter 3) and TRAF2 (Chapter 4).

Taken together, the work presented in this dissertation has elucidated an entirely novel oncogenic signaling network of IKKε-mediated cell transformation in cancer.

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CHAPTER TWO

IKKε-MEDIATED TUMORIGENESIS REQUIRES TRAF2-MEDIATED K63-LINKED POLYUBIUIQINTATION

IKKε-mediated tumorigenesis requires TRAF2-mediated K63-linked polyubiquitination

This chapter is adapted from the following manuscript:

Alicia Y. Zhou, Rhine R. Shen, Eejung Kim, Daqi Tu, Ming Xu, Michael Eck, Zhijian J. Chen, William C. Hahn. IKKε-mediated tumorigenesis requires TRAF2-mediated K63-linked polyubiquitination. *Submitted*

Contributions: Alicia Y. Zhou and William C. Hahn wrote the manuscript. Alicia Y. Zhou, Rhine R. Shen and William C. Hahn conceived and designed the experiments. Alicia Y. Zhou performed all of the experiments with technical assistance from Eejung Kim except Figure2.1.C, which was performed by Rhine R. Shen. Daqi Tu and Michael Eck contributed the crystal structure of TBK1 in Figure 2.10. Ming Xu and Zhijian J. Chen provided the U2OS shUb-Ub(WT) and shUb-Ub(K63R) cell lines.

ABSTRACT

The IκB kinase ε (IKKε, *IKBKE*) is a breast cancer oncogene that is amplified in ~30% of breast cancers and that promotes malignant transformation through the activation of NF-κB signaling. Here, we show that IKKε are modified and regulated by K63-linked polyubiquitination at Lysine 30 and Lysine 401. We found that IKKε K63-linked polyubiquitination is essential for IKKε kinase activity, IKKε-mediated NF-κB activation and IKKε-induced malignant transformation. TRAF2 serves as the K63 ubiquitin E3 ligase that binds to and modifies IKKε. Together, these observations demonstrate that IKKε is regulated by K63-linked ubiquitination in the context of NF-κB-mediated mammary cell transformation.

INTRODUCTION

Nuclear factor κB (NF-κB) signaling plays a critical role in inflammation and innate immunity. Chronic inflammation has long been implicated in the pathogenesis of both solid tumors and blood malignancies [1-3] where aberrant NF-κB signaling in the tumor microenvironment plays an important role [4, 5]. In addition, several studies have also shown that the dysregulation of specific NF-κB family members can contribute to cell transformation in a cell autonomous manner. For example, deletion of the tumor suppressor, *CYLD*, leads to the development of familial cylindromatosis [6-9]. Mutations in the NF-κB effector genes *NFKB1* and *NFKB2* play a role in multiple myeloma [10] and mutations both negative (*A20*) and positive (*CARD11, TRAF2, TRAF5, TAK1* and *RANK*) regulators of NF-κB have been characterized in B-cell lymphoma [11]. More recently, several NF-κB pathway members including *IKBKB, IKBKG, TRAF6,* and *RIP1* have been found to be located in regions of recurrent amplification in epithelial cancers [12].

The canonical NF-κB pathway is activated in response to various proinflammatory signals and converges upon the activation of the IκB kinase (IKK) complex by members of the TRAF E3 ligase family of proteins [2]. The IKK complex consists of two catalytic subunits, IKKα and IKKβ, and the regulatory subunit IKKγ/NEMO [13, 14]. Multiple types of proteasome-dependent and -independent forms of ubiquitination are required for the activation of the NF-κB signaling cascade [15-17]. Several studies demonstrated that the proteasome-independent Lysine63 (K63)-linked ubiquitination of IKKγ is a key step in the activation of the IKK complex [18, 19]. More recent studies have also shown that

linear (Met1) ubiquitination of IKKγ is also sufficient for IKK complex activation [20-22]. The activation of the IKK complex by non-degradative ubiquitination leads to the phosphorylation of the inhibitor of κB (IκB) proteins [23]. This phosphorylation event triggers the Lysine48 (K48)-linked ubiquitination and subsequent preoteasome-mediated degradation of the IκB proteins, which in turn allows for the nuclear translocation of the NF-κB dimers and activation of proimflammatory NF-κB response genes [17].

Inhibitor of κB kinase ε (IKKε, *IKBKE*) is a non-canonical IKK protein that activates interferon, NF-κB and STAT signaling. Together with its binding partner TBK1, IKKε regulates the interferon response pathway to viral challenge by phosphorylation of IRF3 and IRF7 [24-26]. This phosphorylation results in translocation of IRF3 and IRF7 into the nucleus and subsequent activation type I interferon genes [24]. IKKε has also been shown to be a breast cancer oncogene that is amplified and overexpressed in ~30% of breast cancers. Overexpression of *IKBKE* induced malignant transformation of normal epithelial cells in an NF-κB-dependent manner and suppression of IKKε in breast cancer cells that harbor amplifications involving *IKBKE* induced cell death [27]. We have identified the familial tumor suppressor and deubiquitinase CYLD as a direct substrate of IKKε phosphorylation and effector of IKKε-mediated transformation [28]. However, the mechanism(s) that regulate IKKε remain poorly understood.

Here we found that IKKε is K63-ubiquitinated and investigate the role of this modification in IKKε-mediated NF-κB activation and cell transformation.

MATERIALS AND METHODS

Antibodies, Plasmids, and Reagents

The antibodies used include: Myc (clone 4A6) (Millipore), cIAP1, K48-linkage specific Ubiquitin, K63-linkage specific Ubiquitin, phospho-CYLD, TRAF2 (Rabbit) and TANK (Cell Signaling Technologies), V5-HRP (Invitrogen), Ubiquitin (FL-76 and PD-1), β actin-HRP, CYLD, TRAF2 (Mouse) (Santa Cruz Biotechnology), IKKε and (Sigma-Aldrich), HA (Clone12C5) (Boehringer Mannheim). The IKKε phospho-substrate antibody was previously described [28]. Anti-M2 Flag affinity gel sepharose was obtained from Sigma-Aldrich and Glutathione affinity gel sepharose was obtained from GE Healthcare.

pBp Flag-IKKε and Myr-Flag-IKKε were used as described [27]. GST-IKKε and Myc-CYLD was used as described [28]. MF-IKKε K30A, MF-IKKε K401A, MF-IKKε K416A, MF-IKKε K30R, MF-IKKε K401R, MF-IKKε K416R, V5-TBK1 K30R, V5-TBK1 K401R were created using the QuickChange site-directed mutagenesis protocol (Stratagene). V5-TRAF2, V5-TRAF2 ΔRING, V5-IKKε, V5-IKKε K30R, V5-IKKε K401R, V5-IKKε K416R, V5-TBK1, Myc-IKKε, Myc-IKKε K30R, Myc-IKKε K401R, Myc-IKKε K416R were generated by Gateway cloning into the pLEX-V5-Blast lentiviral vector. FLAG-TRAF6 and FLAG-TRAF6 C70A were obtained as a gift from Dr. Hui-Kuan Lin (M.D. Anderson). HA-ubiquitin, HA-Ub K63-only, and HA-Ub K48-only were used as described previously [27, 29]. shRNA constructs were obtained from the RNAi.

Cell Culture, Transfection, Immunoprecipitation, and Immunoblotting. HEK293T, MCF-7 cells were obtained from the American Type Culture Collection (ATCC) and were propagated in DMEM containing 10% FBS. ZR-75-1 cells were obtained from ATCC and propagated in RPMI1640 containing 10% FBS. HA1EM and HMLEM cells have been described [27]. Transfection experiments were performed using Fugene (Roche). U2OS shUb-Ub(WT) and U2OS shUb-Ub(K63R) cells were cultured as previously described [30]. Immunoprecipitations using in U2OS cell lysates were performed as previously described [30]. All other immunoprecipitations and immunoblots were performed as described [28]. Densitometry was assessed using by ImageJ software.

Mass Spectrometry Analysis HEK293T cells were cotransfected with GST-IKKε and HA-Ub. GST immune complexes were isolated using Glutathione Sepharose (GE Healthcare) and the sample was resolved on SDS-PAGE and visualized with Colloidal Blue (Invitrogen). Four bands were excised and subjected to in-gel trypsin digestion. The tryptic peptides were extracted from the gel and analyzed by liquid chromatography MS/MS. Peptides were separated across a 50-min gradient ranging from 7 to 30% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid in a microcapillary (125 μm × 18 cm) column packed with C_{18} reverse-phase material (Magic C18AQ, 5-μm particles, 200-Å pore size, Michrom Bioresources) and analyzed on-line on a hybrid linear ion trap-Orbitrap mass spectrometer (LTQ-Orbitrap, Thermo-Electron). For each cycle, one full MS scan acquired on the Orbitrap at high mass resolution was followed by 10 MS/MS spectra on the linear ion trap from the 10 most abundant ions.

MS/MS spectra were searched using the Sequest algorithm against the human IPI protein database. Dynamic modifications of 114.0429275 Da on lysine was allowed for ubiquitination. All peptide matches were initially filtered based on enzyme specificity, mass measurement error, Xcorr and dCorr scores and further manually validated for peptide identification and site localization.

NF-κB reporter assays

GloResponse NF-κB-RE-*luc2P* HEK293T cells (Promega) were transfected with V5-IKKε WT, V5-IKKε K30R and V5-IKKε K401R. NF-κB activity was measured 36h post-transfection according to the protocol for the One-Glo Luciferase assay (Promega). Luciferase values are reported directly in raw light units (RLU).

Tumorigenicity assay

2 x 10^6 cells were subcutaneously implanted into immunodeficient mice (Balb/c Nude, Charles River Laboratories) anesthetized with isofluorane. Two mice were used per group, and three implantation sites were made per mouse. Tumors were measured at 21 d after implantation.

***In vitro* cell transformation assay**

Growth of HA1EM cells in soft agar was determined by plating 5 X 10^4 cells in triplicate in 0.4% Noble agar. Colonies greater than 100μm in diameter were counted 28 d after plating.

RESULTS

Ubiquitination of IKKε

Since other members of the IKK family are known to be modified and regulated by ubiquitination [15], we tested whether IKKε is ubiquitinated by introducing hemagluttinin (HA)-tagged ubiquitin (HA-Ub) and either Flag-tagged or myristolated-Flag-tagged IKKε (F-IKKε or MF-IKKε) into HEK293T cells. We purified HA immune complexes and found ubiquitinated IKKε species (Figure 2.1A).

To determine whether IKKε ubiquitination occurs under physiological conditions, we investigated IKKε ubiquitination under conditions where IKKε expression induced cell transformation. We previously showed that expression of IKKε confers tumorigenicity to human embryonic kidney (HEK) epithelial and human mammary epithelial cells (HMEC) expressing the SV40 early region (SV40ER), the catalytic subunit of telomerase (*hTERT*) and a constitutively active form of MEK (MEK^{DD}) (Boehm et al., 2007). We isolated IKKε immune complexes from transformed HEK (HA1EM F-IKKε) and HMEC (HMLEM MF-IKKε) cells and found that IKKε is polyubiquitinated in both cell types (Figure. 2.1B and C respectively). We then examined whether IKKε is ubiquitinated in breast cancer cell lines that harbor *IKBKE* amplification and are dependent on IKKε [27]. Specifically, we assessed the status of IKKε ubiquitination in MCF-7 and ZR-75-1 cells and found endogenous polyubiquitinated species of IKKε in both cell lines (Figure 2.1D and E). Together these observations demonstrate that ubiquitination of IKKε occurs in the setting of IKKε-mediated cell transformation.

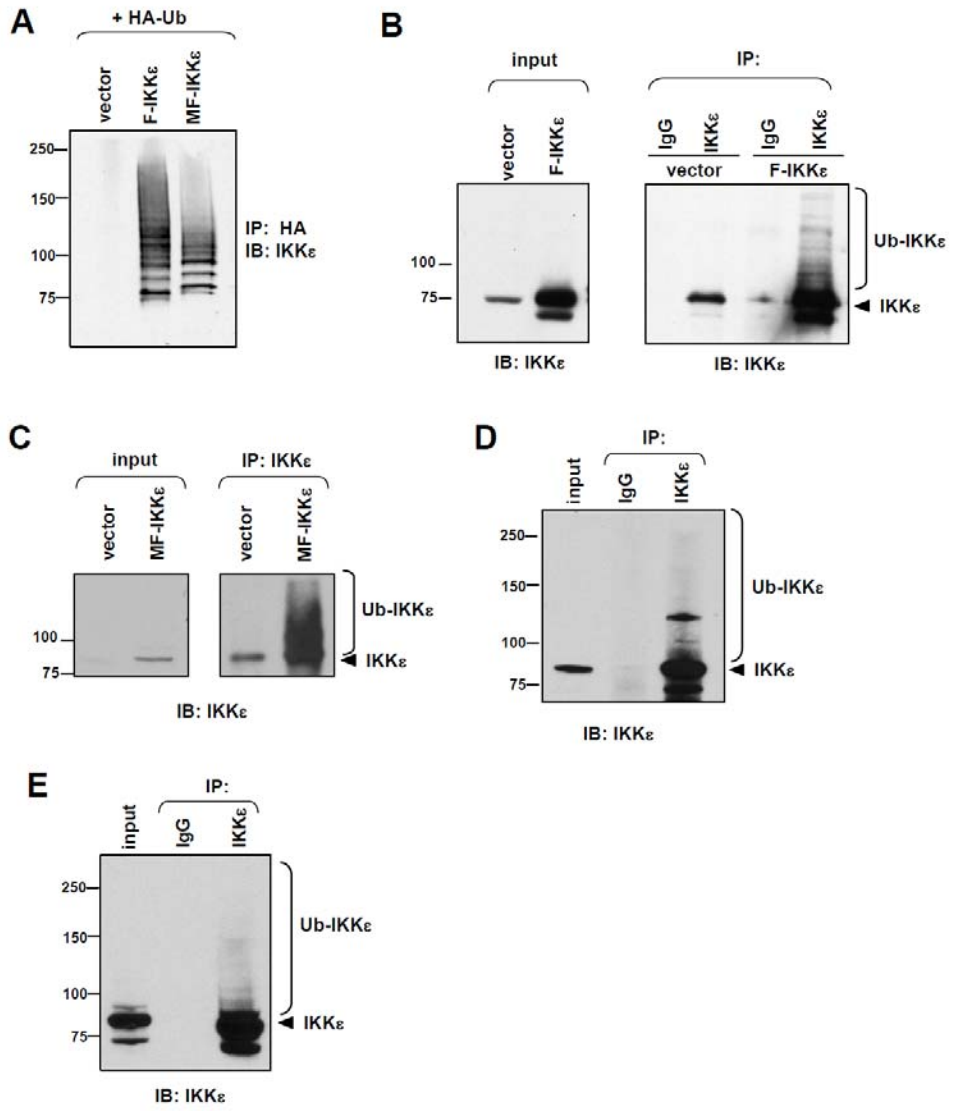


Figure 2.1. IKKε is ubiquitinated in the context of cell transformation
(A) IKKε is ubiquitinated in HEK293T cells. HA immune complexes were isolated from HEK293T cells expressing the indicated proteins and analyzed by immunoblot with an antibody specific for IKKε. (B and C) IKKε is ubiquitinated in transformed cells. IKKε immune complexes were isolated from HA1EM MF-IKKε (B) or HMLEM MF-IKKε (C) cells using an IKKε-specific antibody and analyzed by immunoblot by the same IKKε antibody. 5% of the whole cell lysate was loaded as an input control. Rabbit immunoglobulin (rIgG) was used as a control. (D and E) IKKε is ubiquitinated in breast cancer cell lines. Endogenous IKKε immune complexes were isolated from MCF-7(D) and ZR-75-1(E) breast cancer cell lines using an IKKε-specific antibody and analyzed by immunoblot by the same IKKε antibody. 5% of the whole cell lysate was loaded as an input control.

IKKε undergoes K63-linkage-specific ubiquitination

Whereas Lysine48 (K48)-linked polyubiquitin chains target ubiquitinated substrates to the proteasome for protein degradation, several forms of proteasome- and degradation- independent polyubiquitin chains exist including Lysine63 (K63)-linked, Lysine11 (K11)-linked and linear (Met1) ubiquitin chains [31]. To assess whether IKKε ubiquitination is proteasome-dependent, we treated transformed HA1EM MF-IKKε cells and MCF-7 and ZR-75-1 breast cancer cells with a combination of two proteasome inhibitors, MG-132 and Velcade. As expected, the overall level of ubiquitination was increased in the presence of proteasome inhibitors. However, under these conditions, we failed to observe differences in the level of IKKε protein, suggesting that IKKε ubiquitination does not regulate IKKε stability (Figure 2.2A).

To determine whether IKKε was modified by K48- or K63-linked ubiquitination, we used three methods to determine the type of nature of ubiquitin modification to IKKε. First, we introduced Myc-tagged IKKε along with HA-tagged wildtype, K48-only, or K63-only ubiquitin mutants into HEK293T cells. These mutant HA-ubiquitin constructs can only form either K48-likage-specific (K48-only) or K63-linkage-specific (K63-only) polyubiquitin chains, respectively. We isolated IKKε immune complexes and found that IKKε undergoes robust ubiquitination by wildtype and K63-only ubiquitin (Figure 2.2B). In contrast, we failed to find evidence that IKKε is ubiquitinated by the K48-only ubiquitin mutant (Figure 2.2B).

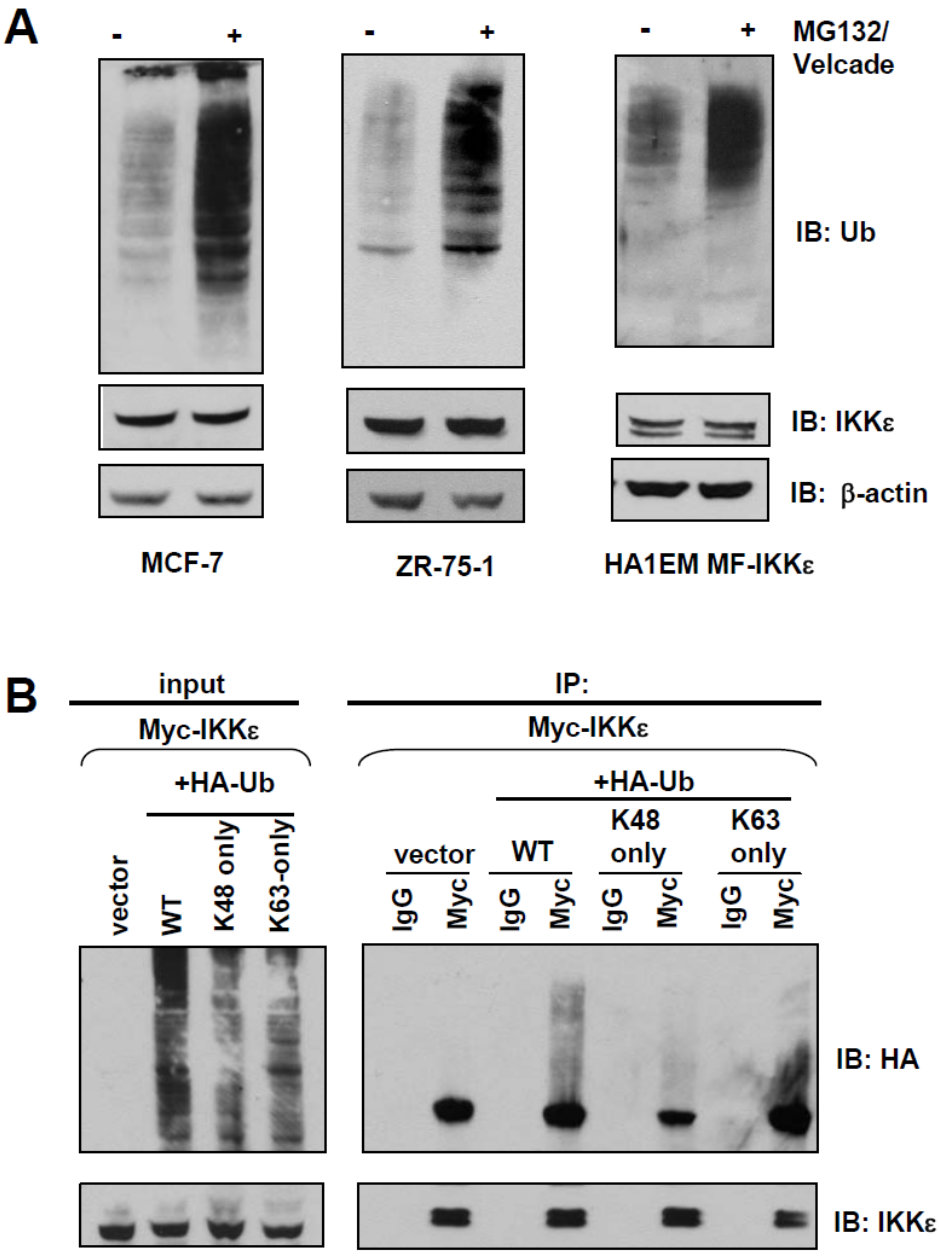


Figure 2.2. IKKε is modified by K63-linked ubiquitination
(A) Proteasome inhibitor treatment does not affect IKKε protein levels. MCF-7 and ZR-75-1 breast cancer cells and HA1EM MF-IKKε transformed cells were subject to treatment with a cocktail of proteasome inhibitors (10μM MG-132, 1μM Velcade) for 4h. Immunoblotting was performed with the indicated antibodies. (B) IKKε undergoes Lys63-linked ubiquitination in HEK293T cells. HEK293T cells were cotransfected as indicated with HA-tagged K63-only or K48-only ubiquitin mutants. Myc immune complexes were isolated followed by immunoblotting with the indicated antibodies. Mouse immunoglobulin (mIgG) was used as a control for immunoprecipitations.

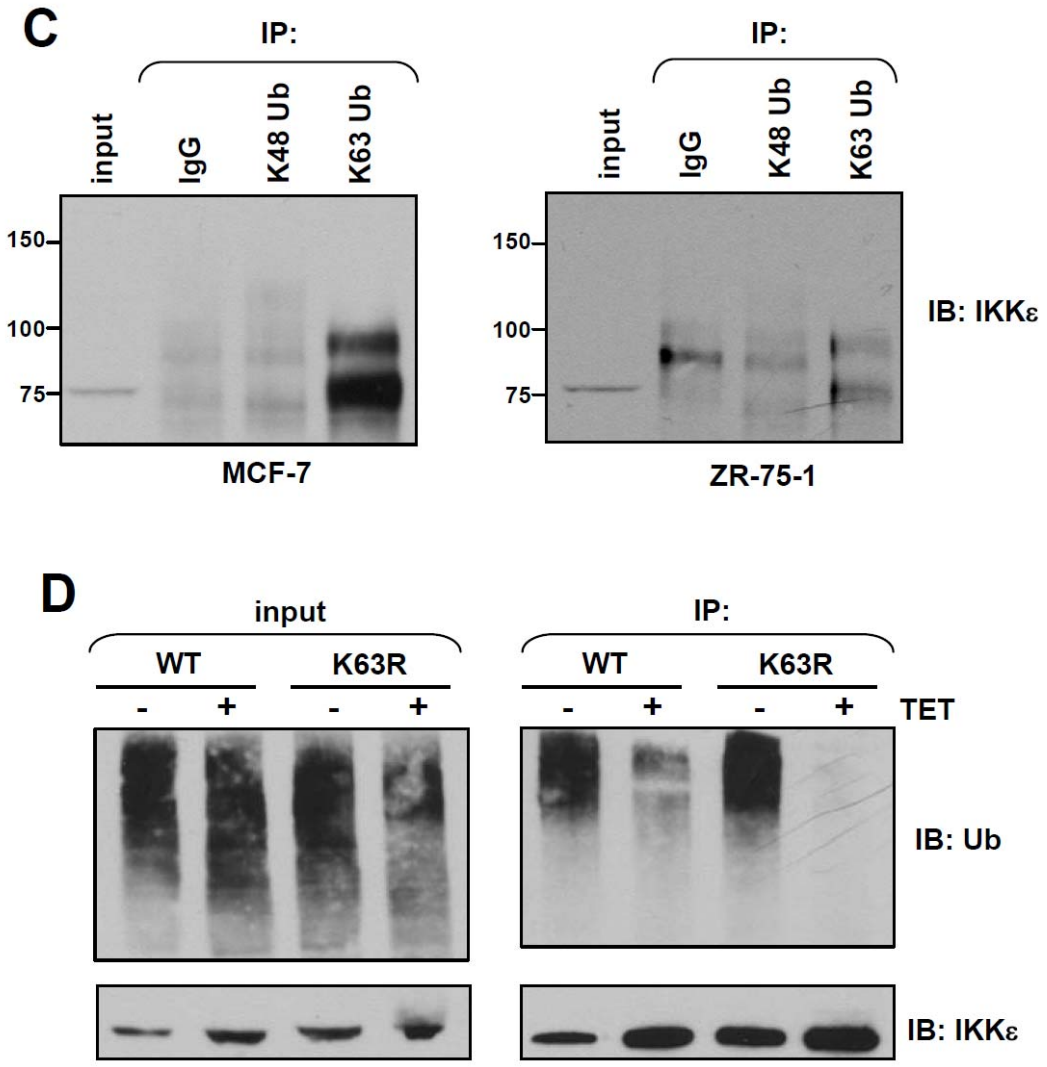


Figure 2.2 Continued

(C) IKKε undergoes Lys63-linked ubiquitination in breast cancer cell lines. Endogenous Lys48-linked (K48) polyubiquitin and Lys63-linked (K63) polyubiquitin immune complexes were isolated followed by immunoblotting with the indicated antibodies in MCF-7 and ZR-75-1 breast cancer cell lines. rIgG was used as a control. (D) U2OS-shUb-Ub(WT) or U2OS-shUb-Ub(K63R) cells were treated with tetracycline (TET) (1 μg/ml) for 4d. IKKε immune complexes were isolated followed by immunoblot analysis with the indicated antibodies. 5% of the whole cell lysate was loaded for comparison (input).

To confirm these observations, we used linkage-specific ubiquitin antibodies. In MCF-7 and ZR-75-1 breast cancer cells, we isolated immune complexes using either a K48- or K63- linkage-specific ubiquitin antibody and found that IKKε was present only in the immune complexes formed by the K63-linkage-specific antibody (Figure 2.2C).

Finally, we used a genetic system in which endogenous forms of ubiquitin are inducibly suppressed by the expression of ubiquitin-specific shRNAs at the same time that wild type or mutant ubiquitin are inducibly expressed in U2OS cells [30]. In the U2OS shUb-Ub(WT) cells, the exogenous construct expresses an shRNA-insensitive wildtype ubiquitin. In the U2OS shUb-Ub(K63R) cells, the exogenous construct expressed an shRNA-insensitive K63R mutant form of ubiquitin – this mutant harbors a lysine-to-arginine mutation at the K63 residue, rendering this ubiquitin incapable of forming K63-linkage-specific chains. Using this system, we isolated IKKε immune complexes from U2OS shUb-Ub(WT) and shUb-Ub(K63R) cells with and without tetracycline treatment and then assessed these complexes for the presence of ubiquitin. We confirmed that IKKε is capable of undergoing modification by the WT ubiquitin chains but is no longer able to be modified by the K63R chains (Figure 2.2D). Taken together, we concluded that IKKε is modified by K63-linked ubiquitin chains in the context of breast cancer cells.

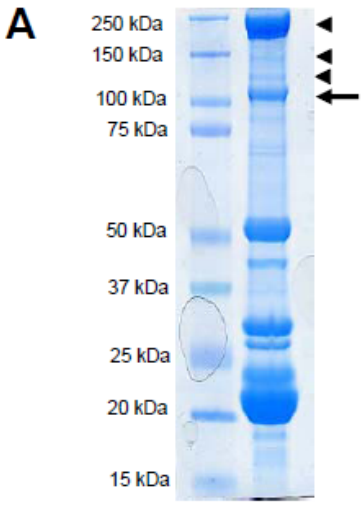
IKKε is ubiquitinated at K30, K401 and K416

To determine the lysine residues on which IKKε is ubiquitinated, we expressed GST-tagged IKKε and HA-tagged ubiquitin in HEK293T cells, isolated

GST immune complexes and performed mass spectrometry. Specifically, we separated GST immune complexes by electrophoresis and submitted four bands for mass spectrometry analysis (Figure 2.3A). We obtained 58.2% coverage of the IKKε protein and 64.7% (22 out of 34) coverage of the internal lysines. From this analysis, three lysine residues of IKKε were identified as ubiquitinated: K30, K401, and K416 (Figure 2.3B, Table 2.1).

To confirm these observations, we generated site-specific lysine-to-alanine (K30A, K401A, K416A) and lysine-to-arginine (K30R, K401R, K416R) IKKε mutants and assessed whether each of these mutants are modified by ubiquitination. We introduced wildtype and mutant IKKε along with HA-ubiquitin into HEK293T cells, isolated IKKε immune complexes and assessed for IKKε ubiquitination. We found that the K30 and K401 mutants exhibited decreased IKKε ubiquitination, but could not detect changes in ubiquitinated species of IKKε K416 mutant (Figure 2.4A). We noted that the lysine-to-arginine and lysine-to-alanine mutants behaved identically in all assays.

We then created stable lines expressing each IKKε mutant (Figure 2.4B) and determined whether these mutants exhibited differential expression of ubiquitinated IKKε species. We found that the K30 and K401 mutant forms of IKKε showed a significant decrease in higher molecular weight ubiquitinated species of IKKε while the K416 mutant showed no signification change (Figure 2.4C). These observations suggest that the K30 and K401 residues of IKKε are essential for IKKε ubiquitination.



B

1 MQSTANYLWH TDDLLGQGAT ASVYKARNKK SGELVAVKVF NTTSYLRPRE
51 VQVREFEVLR KLNHQNIVKL FAVEETGGSR QKVLVMEYCS SGSLLSVLES
101 PENAFGLPED EFLVVLRCVV AGMNHLRENG IVHRDIKPGN IMRLVGEEGQ
151 SIYKLTDFGA ARELDDDEKF VSVYGTEEYL HPDMYERAVL RKPQQKAFGV
201 TVDLWSIGVT LYHAATGSLP FIPFGGPRRN KEIMYRITTE KPAGAIAGAQ
251 RRENGPLEWS YTLPITCQLS LGLQSQLVPI LANILEVEQA KCWGFDQFFA
301 ETSDILQRVV VHVFSLSQAV LHHIYIHAHN TIAIFQEAVH KQTSVAPRHQ
351 EYLFEGHLCV LEPSVSAQHI AHTTASSPLT LFSTAIPKGL AFRDPALDVP
401 KFVPKVDLQA DYNTAKGVLG AGYQALRLAR ALLDGQELMF RGLHWVMEVL
451 QATCRRTLEV ARTSLLYLSS SLGTERFSSV AGTPEIQELK AAAELRSRLR
501 TLAEVLSRCS QNITETQESL SSLNRELVKS RDQVHEDRSI QQIQCCLDKM
551 NFIYKQFKKS RMRPGLGYNE EQIHKLDKVN FSHLAKRLLQ VFQEECVQKY
601 QASLVTHGKR MRVVHETRNH LRLVGCSVAA CNTEAQGVQE SLSKLLEELS
651 HQLLQDRAKG AQASPPPIAP YPSPTRKDLL LHMQELCEGM KLLASDLLDN
701 NRIIERLNRV PAPPDV

Figure 2.3. Identification of IKKε ubiquitination residues.
(A)Ubiquitinated IKKε analysis by mass spectrometry. GST-IKKε was cotransfected into HEK293T cells with HA-Ub. GST immune complexes were isolated and subjected to SDS-PAGE followed by Colloidal Blue staining. The band corresponding to IKKε (arrow) and 3 additional higher molecular weight bands (arrowheads) were excised from the gel and digested with trypsin and chymotrypsin. Ubiquitination sites were mapped by microcapillary LC/MS/MS. (B) Amino acid sequence of IKKε. Mass spectrometry analysis covered 58.2% of the IKKε amino acid sequence (underlined) and 64.7% (22/34) of the internal lysines (bold). Three lysines were identified as ubiquitinated K30, K401 and K416 (red).

Table 2.1. Mass spectrometry analysis of IKKε ubiquitination

POSITION	MH+	PEPTIDE SEQUENCE
30-38	**931.1204**	**K#SGELVAVK**
30-38	931.1204	KSGELVAVK
31-38	802.9463	SGELVAVK
39-49	1354.5517	VFNTTSYLRPR
61-69	1094.3022	KLNHQNIVK
62-69	966.1281	LNHQNIVK
70-80	1166.2762	LFAVEETGGSR
118-127	1100.3423	CVVAGMNHLR
144-154	1223.3684	LVGEEGQSIYK
155-162	850.9499	LTDFGAAR
163-187	3081.2909	ELDDDEKFVSVYGTEEYLHPDMYER
230-236	954.1321	NKEIMYR
237-251	1484.6957	ITTEKPAGAIAGAQR
292-308	2064.2794	CWGFDQFFAETSDILQR
389-401	1399.6329	GLAFRDPALDVPK
394-401	854.9787	DPALDVPK
394-405	**1326.5787**	**DPALDVPK#FVPK**
406-416	1238.3397	VDLQADYNTAK
406-427	**2324.5986**	**VDLQADYNTAK#GVLGAGYQALR**
417-427	1105.2821	GVLGAGYQALR
431-441	1293.5236	ALLDGQELMFR
442-455	1643.9624	GLHWVMEVLQATCR
456-462	844.9896	RTLEVAR
463-476	1527.7147	TSLLYLSSSLGTER
477-490	1506.6959	FSSVAGTPEIQELK
501-508	889.0397	TLAEVLSR
509-525	1911.0507	CSQNITETQESLSSLNR
530-538	1142.1735	SRDQVHEDR
532-538	898.9078	DQVHEDR
539-549	1279.5120	SIQQIQCCLDK
550-555	816.0057	MNFIYK
562-575	1672.8993	MRPGLGYNEEQIHK
562-578	2029.3214	MRPGLGYNEEQIHKLDK
576-586	1272.4899	LDKVNFSHLAK
579-586	916.0678	VNFSHLAK
587-599	1620.9073	RLLQVFQEECVQK
588-599	1464.7198	LLQVFQEECVQK
600-609	1104.2511	YQASLVTHGK
623-644	2195.4676	LVGCSVAACNTEAQGVQESLSK
645-657	1594.8080	LLEELSHQLLQDR
658-676	1907.1797	AKGAQASPPPIAPYPSPTR
660-676	1707.9268	GAQASPPPIAPYPSPTR
660-677	1836.1009	GAQASPPPIAPYPSPTRK
678-691	1661.0022	DLLLHMQELCEGMK
692-702	1244.3901	LLASDLLDNNR
707-716	1078.2566	LNRVPAPPDV

Table 2.1. Mass spectrometry analysis of ubiquitinated IKKε. All the IKKε peptides that were identified in the mass spectrometry analysis are listed in increasing positional order and corresponding molecular mass and peptide sequence. Peptides that correspond to ubiquitinated residues of IKKε are highlighted (bold).

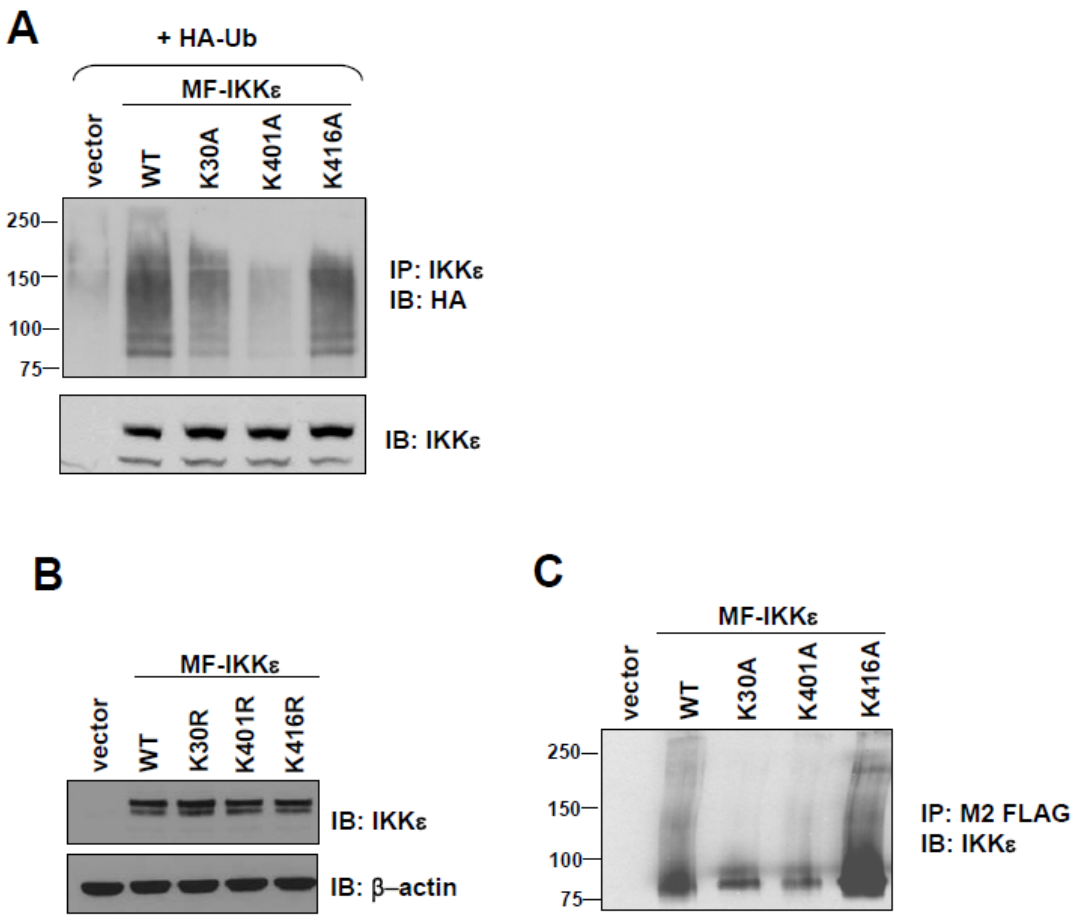


Figure 2.4. IKKε is ubiquitinated on K30, K401 and K416.
(A) IKKε ubiquitination site mutants in HEK293T cells. Site-directed MF-IKKε mutants were created in which K30, K401 and K416 residues were changed to an alanine. These mutants were cotransfected into HEK293T cells with HA-Ub. IKKε immune complexes were isolated with an IKKε-specific antibody and analyzed by immunoblotting. (B) IKKε ubiquitination site mutants in transformed HA1EM cells. Site-directed MF-IKKε mutants were created in which K30, K401 and K416 residues were changed to an arginine. These mutants were retrovirally introduced into HA1EM cells. (C) Mutant IKKε ubiquitination status in transformed HA1EM cells. IKKε immune complexes were isolated from HA1EM cells expressing wildtype, K30A, K401A and K416A MF-IKKε with Anti-M2 Flag Sepharose and analyzed by immunoblotting.

IKKε ubiquitination at K30 and K401 are essential for IKKε kinase activity

We previously identified the deubiquitinase CYLD as a downstream kinase target of IKKε [28]. To determine the importance of IKKε K63-linked ubiquitination on IKKε kinase function, we used our genetic ubiquitin replacement system. We isolated CYLD immune complexes from U2OS shUb-Ub(WT) and shUb-Ub(K63R) cells with and without tetracycline treatment and determined the levels of both phospho (pCYLD) - and total CYLD protein. We found that in the conditions under which IKKε cannot be K63-linked ubiquitinated, IKKε also exhibited impaired kinase activity (Figure 2.5A). Specifically, we found that under conditions in which IKKε was not ubiquitinated, we failed to detect phosphorylation of CYLD as assessed by a phosphospecific antibody directed to pCYLD and alterations in CYLD mobility.

To determine the effect of the K30R and K401R mutants on IKKε kinase activity, we assessed the ability of wildtype and mutant IKKε to phosphorylate CYLD *in vivo*. We transiently cotransfected HEK293T cells with Myc-tagged CYLD and either WT, K30R, or K401R IKKε. We isolated Myc immune complexes and determined CYLD phosphorylation status by IKKε pSubstrate immunoblot. These observations confirmed that although wildtype IKKε phosphorylates CYLD, neither of the mutant forms of IKKε exhibited kinase activity (Figure 2.5B). We note that we found both IKKε mutants in CYLD immune complexes, indicating that despite lacking enzymatic activity, these mutants retained the ability to form a complex with CYLD.

IKKε activates the NF-κB pathway through a non-conventional mechanism [32], and this activity is essential for IKKε-mediated transformation [27]. To assess

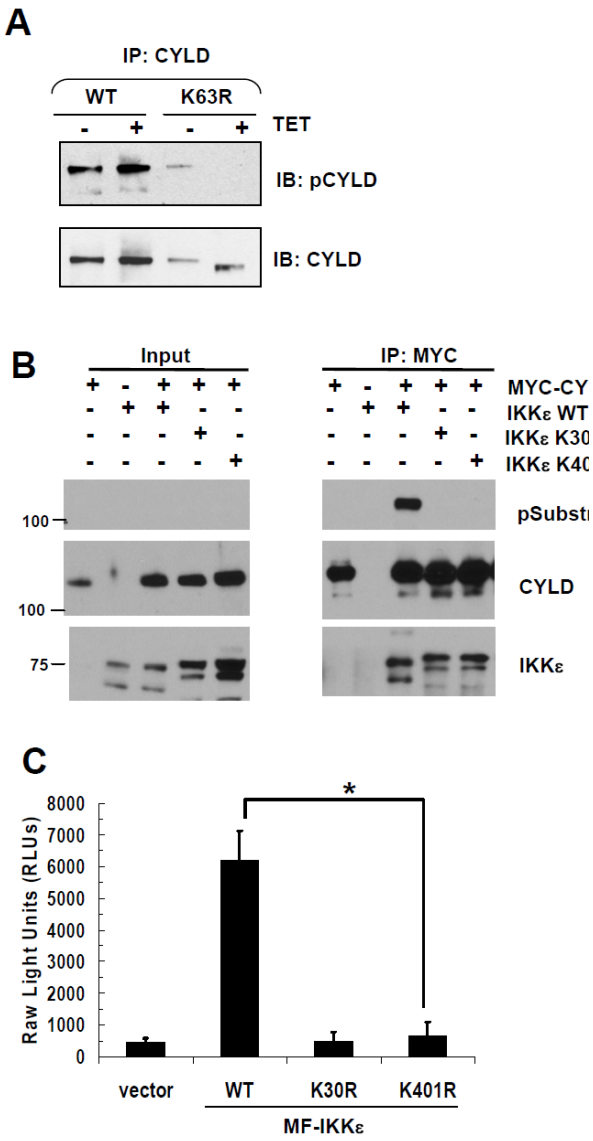


Figure 2.5. IKKε K63-linked ubiquitination at K30 and K401 is essential for IKKε function

(A) IKKε kinase function is dependent on its K63-linked ubiquitination. U2OS-shUb-Ub(WT) or U2OS-shUb-Ub(K63R) cells were treated with tetracycline (TET) (1 μg/ml). CYLD immune complexes were isolated followed by immunoblot analysis with antibodies for phospho-CYLD and total CYLD. rIgG was used for control immunoprecipitations. (B) Effects of IKKε ubiquitination mutants on CYLD phosphorylation. HEK293T cells were transfected as indicated. Myc-CYLD immune complexes were isolated and analyzed with antibodies specific for CYLD, IKKε, and IKKε phospho-substrates. (C) Effects of IKKε ubiquitination mutants on NF-κB activation. GloResponse NF-κB-RE-*luc2P* HEK293T cells were transfected with V5-IKKε WT, V5-IKKε K30R and V5-IKKε K401R and analyzed by the One-Glo Luciferase assay. Raw light unit (RLU) activities are reported. Results reported as mean ± SD of six experiments. *$p = 1.1 \times 10^{-7}$, calculated by a standard t test.

the effects of disrupting IKKε ubiquitination on NF-κB activation, we examined whether the IKKε mutants exhibited activity in a NF-κB luciferase reporter assay (Figure 2.5C). Consistent with the kinase activity data, these results indicated that WT but not K30R or K401R mutant IKKε was able to induce the NF-κB luciferase reporter activity.

IKKε ubiquitination at K30 and K401 are essential for IKKε-mediated transformation

To interrogate the role of ubiquitination in IKKε-mediated cell transformation, we assessed whether mutant forms of IKKε were able to transform human cells (Figure 2.6A). In HA1EM cells, expression of wild type IKKε induces robust anchorage independent colony growth. In contrast, when we expressed the K30 and K401 IKKε mutants, we found that these mutants were markedly defective in their ability to induce anchorage independent colony growth. This transformation phenotype was identical in both the lysine-to-arginine and lysine-to-alanine mutants (Figure 2.6B).

To confirm these *in vitro* findings, we then assessed whether expression of WT or mutant IKKε conferred tumorigenicity. We found that WT IKKε induced tumor formation at 50% incidence, which is consistent with previous published data [27]. In contrast expression of the K30 and K401 IKKε mutants exhibited an impaired tumorigenic potential (Figure 2.6C). These observations indicate that the K63-

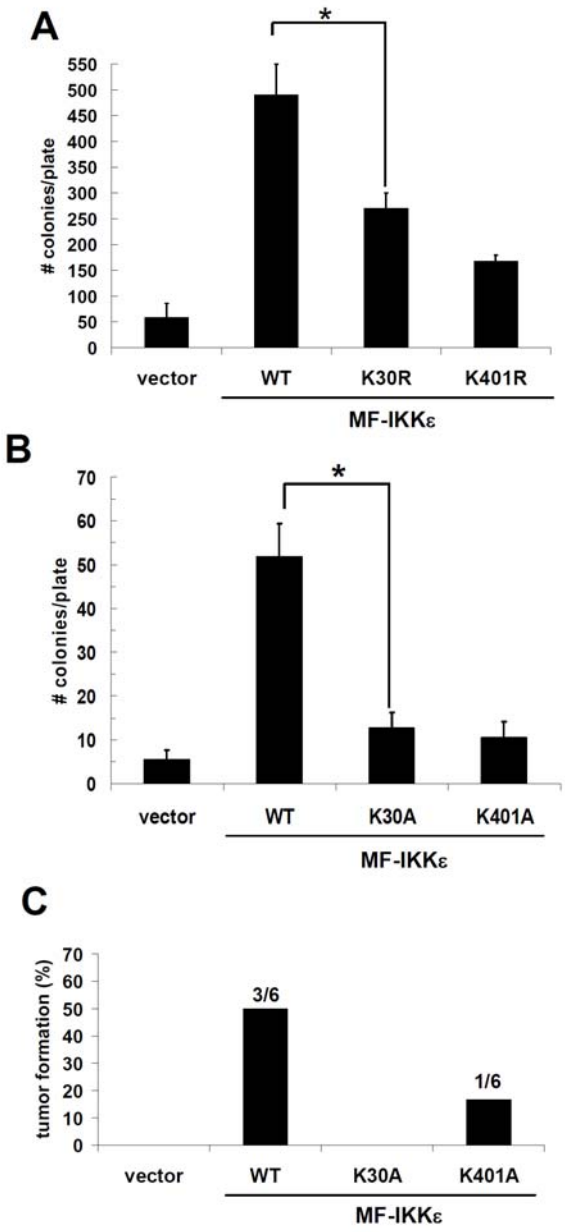


Figure 2.6. IKKε ubiquitination at K30 and K401 is essential for IKKε-mediated transformation

(A) Anchorage-independent growth of HA1EM cells transformed with wildtype and mutant IKKε. Colony formation of cells from was analyzed after 28 d. Results reported as mean ± SD of three experiments. *p = 0.0045, calculated by a standard t test. (B) Anchorage-independent growth of HA1EM cells transformed with wildtype and mutant lysine-to-alanine IKKε. Colony formation of cells from was analyzed after 28 d. Results reported as mean ± SD of three experiments. * $p = 3.9 \times 10^{-7}$, calculated by a standard t test. (C) Tumorigenicity of HA1EM MF-IKKε wildtype and mutant cells. HA1EM cells expressing wildtype or mutant IKKε were introduced subcutaneously into immunodeficient mice. Tumor formation shown as a percentage assessed at 21 days postinjection.

linkage-specific ubiquitination of IKKε at K30 and K401 are essential for the ability of IKKε to mediate malignant cell transformation.

TRAF2 associates with and ubiquitinates IKKε

Having defined a role for K63-linked ubiquitination in the regulation of IKKε in both the context of NF-κB activation and cell transformation, we sought to identify the E3 ligase responsible for IKKε ubiquitination. IKKε forms a complex that includes TBK1, TRAF2, cIAP-1 and TANK in the context of innate responses to viral challenge [33, 34]. TRAF2 has been shown to be the E3 ligase that promotes the K63-linked ubiquitination of RIP1 during activation of the canonical TNF receptor-induced NF-κB pathway [35-37]. Based on these observations, we tested whether TRAF2 serves as an E3 ligase complex to modify IKKε.

To confirm that IKKε interacts with TRAF2, we isolated IKKε immune complexes in the MCF-7 and ZR-75-1 breast cancer cell lines. In both cell lines, we confirmed that IKKε binds to TRAF2 in transformed cells (Figure 2.7A). To determine if TRAF2 catalyzes IKKε ubiquitination, we introduced IKKε and either a wildtype or an E3 ligase-deficient mutant TRAF2 (TRAF2 ΔRING) into HEK293T cells. The TRAF2 ΔRING construct harbors an 87 amino acid N-terminal deletion of the conserved RING domain, which inactivates its E3 ligase activity [37]. In these cells, we assessed whether IKKε was ubiquitinated in the presence of TRAF2 and found that wildtype TRAF2 robustly catalyzed the polyubiquitination of IKKε. In

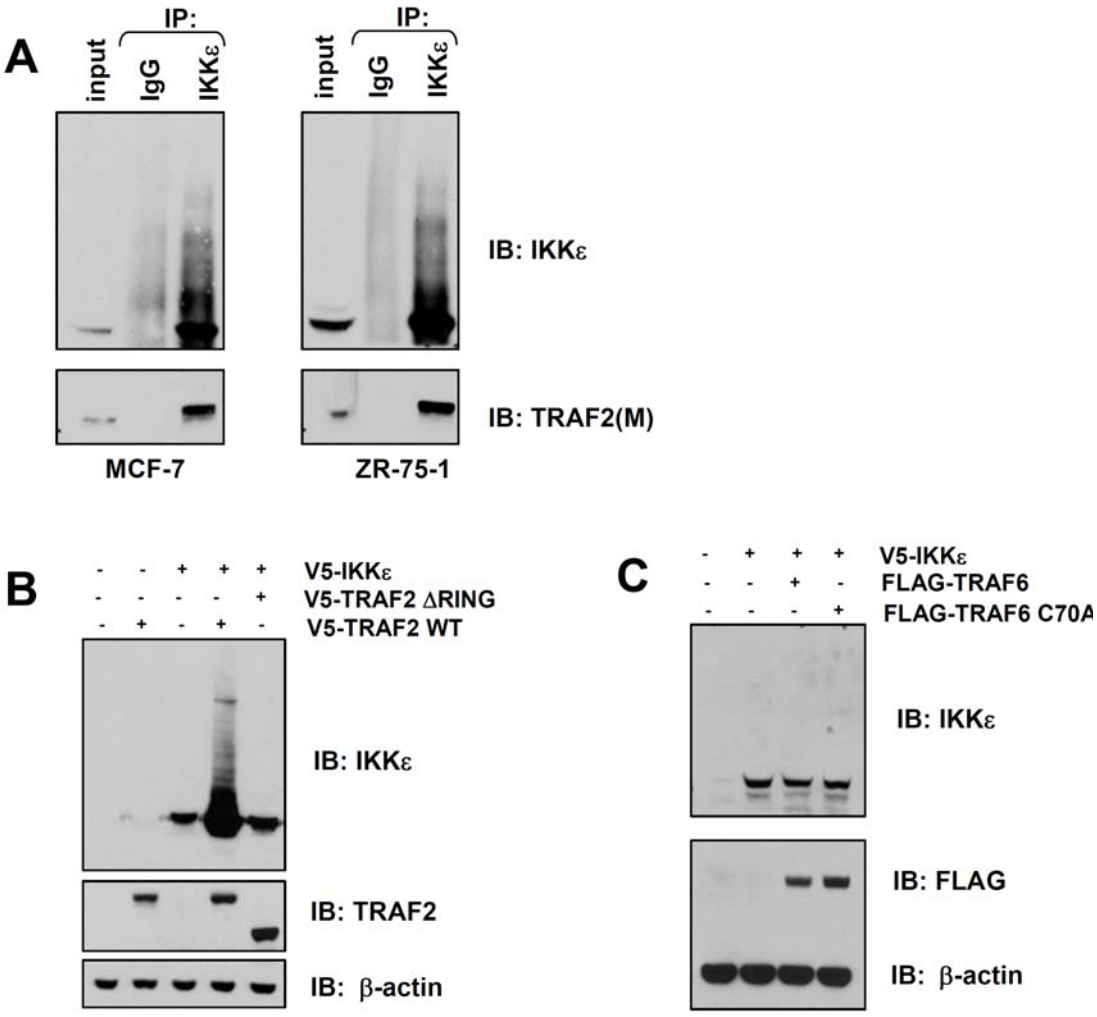


Figure 2.7. TRAF2 is a K63-linked ubiquitin ligase that modifies IKKε
(A) IKKε binds to TRAF2 in breast cancer cell lines. Endogenous IKKε immune complexes were isolated from MCF-7 and ZR-75-1 breast cancer cell lines using an IKKε-specific antibody and analyzed by immunoblotting. rIgG was used as a control. (B) TRAF2 ubiquitinates IKKε. HEK293T cells were transfected as indicated. Lysates were analyzed by immunoblot with antibodies for IKKε, β-actin and TRAF2. (C) TRAF6 does not ubiquitinate IKKε. HEK293T cells were transfected as indicated. Lysates were analyzed by immunoblot with antibodies for IKKε, β-actin TRAF6

contrast, we failed to find polyubiquitinated forms of IKKε in cells co-expressing IKKε and the TRAF2 ΔRING mutant (Figure 2.7B).

To confirm the specificity of TRAF2 for IKKε, we tested whether expression of a different TRAF family member, TRAF6, which has been shown to catalyze the K63-linked polyubuiqitination of Akt [38], induced IKKε ubiquitination. We found that neither wildtype nor catalytically inactive (C70A) TRAF6 was capable of catalyzing IKKε ubiquitination (Figure 2.7C). These observations provided evidence that TRAF2, but not TRAF6, catalyzed IKKε polyubiquitination and that the RING domain of TRAF2 is required for this activity.

To determine whether TRAF2 is required for IKKε ubiquitination, we suppressed TRAF2 expression using two independent *TRAF2*-specific shRNAs (shTRAF2 #1 and shTRAF2 #2) in MCF-7 and ZR-75-1 breast cancer cells. These *TRAF2*-specific shRNAs suppressed the levels of TRAF2 expression in both MCF-7 and ZR-75-1 cells (Figure 2.8A and 2.8B respectively). We then isolated IKKε immune complexes from these cells and found that both MCF-7 and ZR-75-1 cells in which TRAF2 was suppressed exhibited a decreased level of polyubiquitinated IKKε (Figure 2.8C and 2.8D respectively). We next used the K63-linkage specific antibody to isolate immune complexes and found that in cells with suppressed TRAF2 had decreased amounts of K63-ubiquitin modified IKKε in both MCF-7 and ZR-75-1 breast cancer cells (Figure 2.8E and 2.8F respectively). These observations provide evidence that TRAF2 is a K63-linkage E3 ubiquitin ligase enzyme that associates with IKKε.

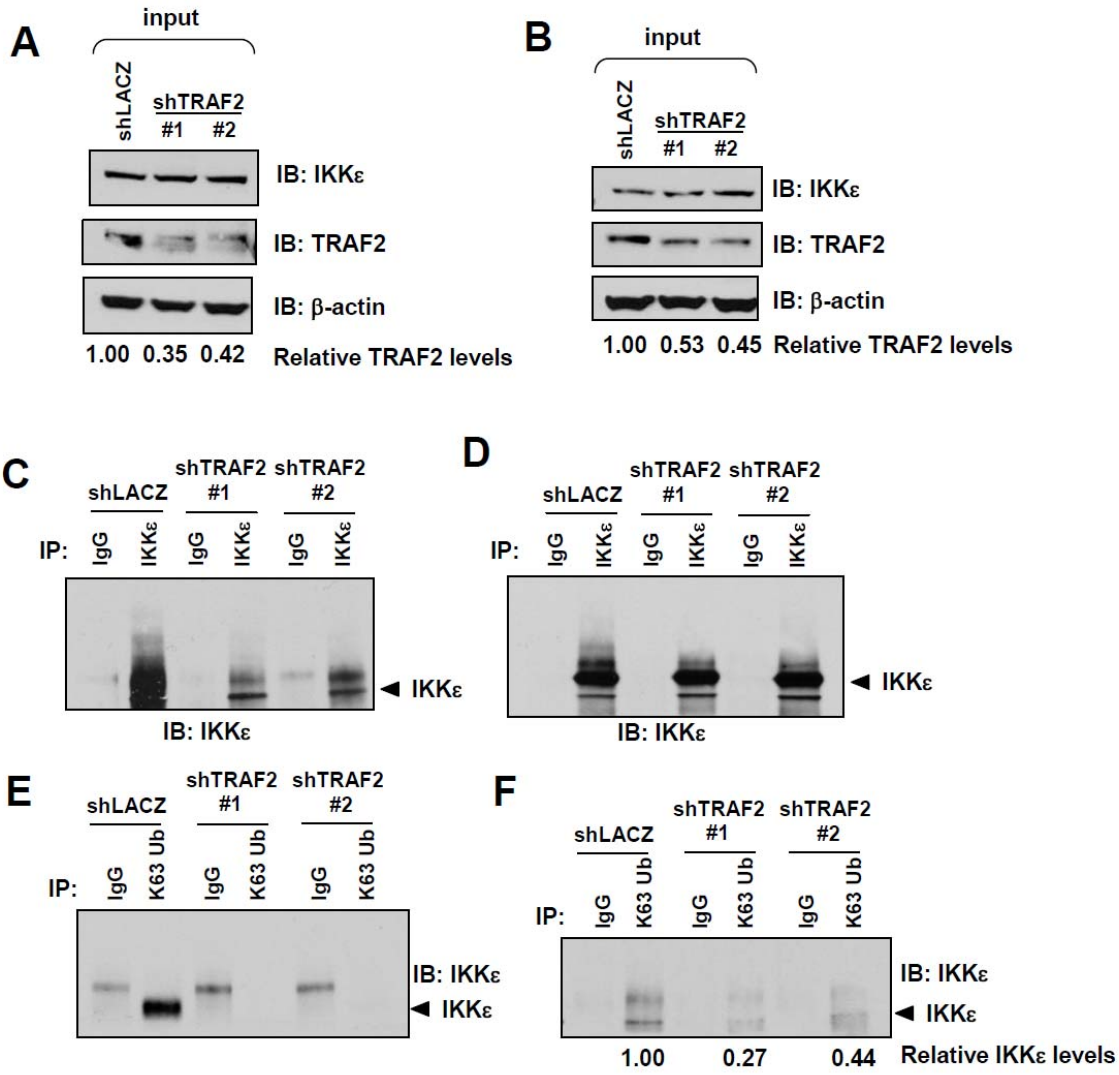


Figure 2.8. TRAF2 suppression reduces IKKε K63-linked ubiquitination
(A and B) Effects of *TRAF2* suppression in MCF-7(A) and ZR-75-1(B) cells. MCF-7 and ZR-75-1 cells were transduced with shTRAF2#1, shTRAF2#2 or control shLACZ. Lysates were analyzed by immunoblot with antibodies for IKKε, TRAF2 and β-actin. Relative TRAF2 levels were calculated by densitometry analysis. (C and D) IKKε immune complexes were isolated from MCF-7(C) and ZR-75-1(D) cells with *TRAF2* suppression with an IKKε-specific antibody and analyzed by immunoblot with an IKKε antibody. rIgG was used for control immunoprecipitations. (E and F) K63-linkage ubiquitin immune complexes were isolated from MCF-7(E) and ZR-75-1(F) cells with *TRAF2* suppression with an K63-linkage-specific ubiquitin antibody and analyzed by immunoblot with an IKKε antibody. rIgG was used for control immunoprecipitations. Relative IKKε levels were calculated by densitometry analysis.

TBK1 undergoes K63-linked ubiquitination at K30 and K401

IKKε shares 65% similarity to another non-canonical IKK protein, TANK-binding kinase 1 (TBK1) [39]. In the context of innate immunity, IKKε and TBK1 work in complex to activate the interferon response. Given the structural and functional similarity between these two kinases, we next explored whether TBK1 is also regulated by K63-linked ubiquitination.

To determine if TBK1 undergoes K63-linked ubiquitination, we introduced GST-tagged TBK1 along with HA-tagged wildtype or K63-only ubiquitin mutants into HEK293T cells. We isolated GST immune complexes and found that TBK1 undergoes robust ubiquitination by wildtype and K63-only ubiquitin (Figure 2.9A). Interestingly, the K30 and K401 are conserved between the IKKε and TBK1 protein sequence. Since our previous results indicated that IKKε was ubiquitinated at these two residues, we next interrogated whether TBK1 was also ubiquitinated at these residues. We generated site-specific lysine-to-arginine (K30R, K401R) TBK1 mutants and assessed whether each of these mutants is capable of undergoing K63-linked ubiquitination. We introduced wildtype and mutant V5-tagged TBK1 along with K63-only HA-ubiquitin into HEK293T cells. We isolated V5 immune complexes and assessed TBK1 ubiquitination. We found that, similar to IKKε, the K30 and K401 TBK1 mutants exhibited a decrease in ubiquitination (Figure 2.9B). Taken together, this data is evidence that TBK1 is also regulated by K63-linked ubiquitination at K30 and K401.

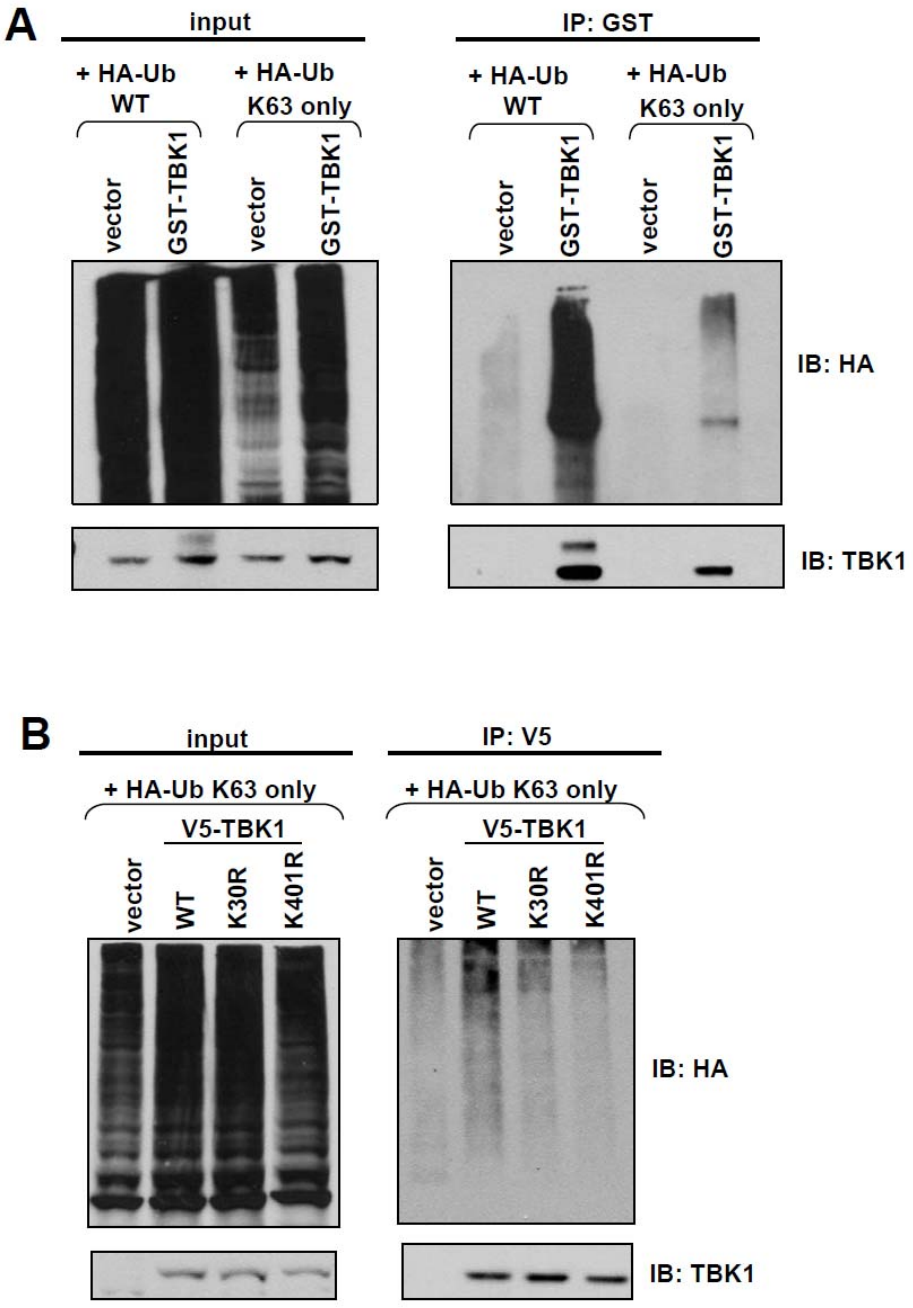


Figure 2.9. TBK1 undergoes K63-linked ubiquitination
(A) HEK293T cells were cotransfected as indicated with HA-tagged wildtype or K63-only ubiquitin. GST immune complexes were isolated followed by immunoblotting with the indicated antibodies. (B) TBK1 ubiquitination site mutants in HEK293T cells. Site-directed V5-TBK1 mutants were created in which K30 and K401 residues were changed to an arginine. These mutants were cotransfected into HEK293T cells with HA-Ub K63 only. V5 immune complexes were isolated with and analyzed by immunoblotting with indicated antibodies.

DISCUSSION

K63-linked ubiquitination of IKKε is essential for its activity as an oncogene

In the context of innate immunity, IKKε plays a key regulatory role in initiating the interferon response to viral challenge. In addition, IKKε has been identified as a breast cancer oncogene that is amplified and overexpressed in ~30% of breast cancers. Breast cancer cell lines that harbor amplifications involving *IKBKE* are dependent on IKKε expression for survival. Thus, understanding the mechanism of IKKε regulation and function is an important key step towards elucidating its roles not only in these biological processes but also in the context of malignant disease.

Here, we have used a combination of biochemical assays and an experimental model system of defined genetic composition to demonstrate that IKKε is specifically modified by K63-linked ubiquitination. Through a large-scale mass spectrometry approach, we have identified IKKε residues that are modified by ubiquitination. Using mutational analysis, we have determined that K30 and K401 are important sites of IKKε modification and that the ubiquitination of IKKε on these two sites is essential for its ability to act both as an NF-κB activator and as a breast cancer oncogene.

In prior work, we showed that IKKε induces cell transformation in a manner that is dependent upon activation of the NF-κB pathway. Here we show that K63-linked ubiquitination of IKKε is required for IKKε kinase activity, suggesting that this modification induces either an allosteric or complex formation change to regulate kinase activity. Since IKKε also regulates interferon, STAT and NF-κB signaling in

the context of viral challenge or other pro-inflammatory stimuli [40], further studies are necessary to dissect the role of K63-linked ubiquitination in these settings.

We found that ubiquitination of both K30 or K401 are required for kinase activity. Since these mutants exhibit equivalent binding to CYLD, it is unlikely that these mutants disrupt the overall structure of IKKε. Here, we have also shown that the analogous residues in TBK1 that we identified as ubiquitinated in IKKε are also ubiquitinated in TBK1. Work in the Eck lab has described the structure of TBK1 (unpublished). Upon mapping of the residues K30 and K401 onto the TBK1 crystal structure, we found that these residues reside on opposing faces of the protein structure but are in close approximation when TBK1 forms a homodimer (Figure 2.10). This suggests that these residues may be important for protein interactions by an E3 ligase or other interacting partner with this face of the homodimer and could be a mechanistic explanation for the essentiality of these residues in downstream protein function. Our work here has preliminarily defined a role for ubiquitination in the role of TBK1 regulation, more function experiments are required to determine if TBK1 is regulated by ubiquitination in the same way and to the same degree as IKKε.

TRAF2 is an E3-ubiqutin ligase that interacts and modifies IKKε

We found that the IKKε-interacting protein and RING finger domain protein TRAF2 catalyzed IKKε ubiquitination in a manner dependent on the TRAF2 RING domain. Moreover, we found that suppression of TRAF2 decreased IKKε ubiquitination, indicating that TRAF2 plays an essential role in the modification of IKKε. These observations implicate TRAF2 as an E3 ubiquitin ligase for IKKε.

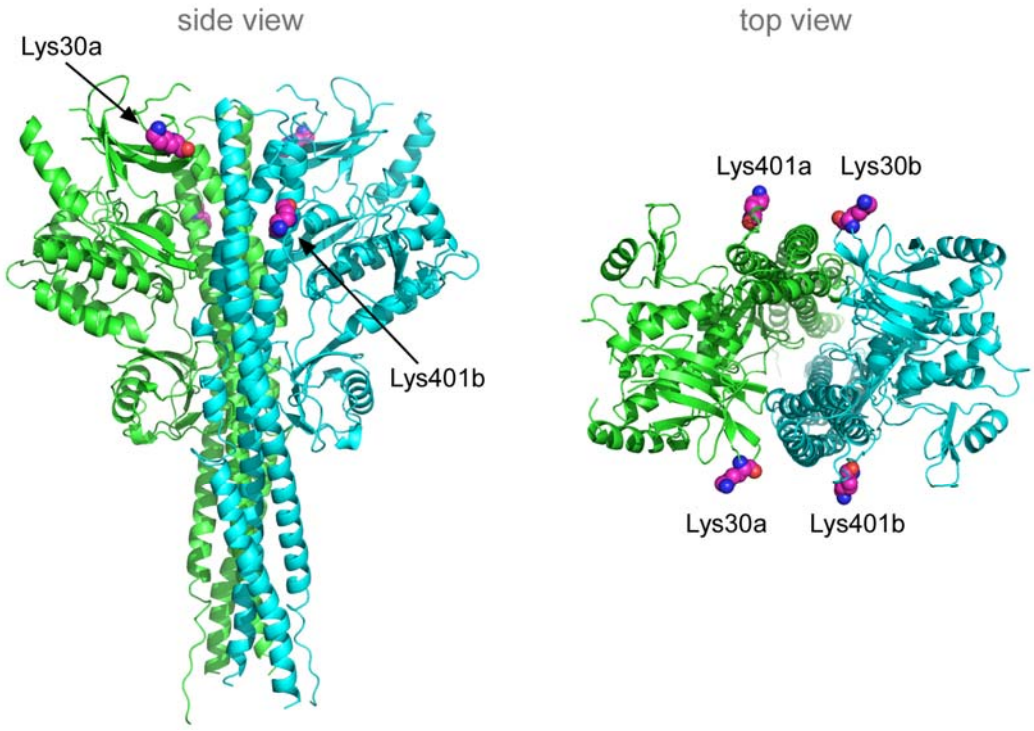


Figure 2.10. Structure of TBK1 homodimer
Overall structure of the TBK1 dimer. One molecule is colored in green(a) and one TBK1 molecule is colored in blue(b). Lys30 and Lys401 are shown on both molecules.

TRAF2 is also known to interact in the same complex with TBK1, cIAP1, and TANK [33, 34]. Previously, TRAF2 has also been characterized in complex with RIP1 and cIAP1 to act as the E3 ligase that is responsible for RIP1 K63-linked ubiquitination [37]. Other studies have also shown cIAP1 and cIAP2 to also catalyze RIP1 ubiquitination [41]. Our work here has demonstrated that TRAF2, but not TRAF6, is required for IKKε K63-linked ubiquitination in context of cancer. However, with ~600 E3 ubiquitin ligases in the human genome, further work is needed to determine if there are any other E3 ligases whose function may be sufficient to catalyze IKKε ubiquitination.

Although IKKε is a serine-threonine kinase potentially amenable to inhibition by small molecule inhibitors, developing inhibitors that specifically target particular kinases remains a challenge in the field. Our work here identifies TRAF2-mediated K63-linked ubiquitination as essential for IKKε regulation and function. Recent work has proven that E3 ligases can also be targeted and inhibited by small molecule inhibitors [42]. In particular, small molecule inhibitors of the cullin-RING family of E3 ubiquitin ligases have been described [43, 44]. Thus, these observations not only provide new insights into the regulation of IKKε but may also identify an alternative mechanism to target IKKε therapeutically.

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CHAPTER THREE

PHOSPHORYLATION OF THE TUMOR SUPPRESSOR CYLD BY THE BREAST CANCER ONCOGENE IKKε PROMOTES CELL TRANSFORMATION

Phosphorylation of the tumor suppressor CYLD by the breast cancer oncogene IKKε promotes cell transformation

This chapter is adapted from the following publication:

Jessica E. Hutti, Rhine R. Shen, Derek W. Abbott, Alicia Y. Zhou, Kam M. Sprott, John M. Asara, William C. Hahn, and Lewis C. Cantley. Phosphorylation of the Tumor Suppressor CYLD by the Breast Cancer Oncogene IKKε Promotes Cell Transformation. *Mol Cell.* 2009 May 14;34(4):461-72.

The published article is included in Appendix 1.

Contributions: Jessica E. Hutti, Rhine R. Shen, William C. Hahn and Lewis C. Cantley wrote the manuscript. Jessica E. Hutti, Rhine R. Shen, William C. Hahn and Lewis C. Cantley conceived and designed the experiments. Jessica E. Hutti and Derek W. Abbott performed the unbiased peptide scanning library screen. Jessica E. Hutti performed the biochemical experiments with technical assistance from John M. Asara. Rhine R. Shen and Alicia Y. Zhou performed the functional and transformation assay experiments. Kam M. Sprott produced the IKKε P-substrate-specific antibody. Specific contributions: Alicia Y. Zhou produced the data shown in Figure 3.1A , 3.1B. and 3.6C and adapted the manuscript for this chapter.

ABSTRACT

The non-canonical IKK family member IKKε is essential for regulating antiviral signaling pathways and is a recently discovered breast cancer oncoprotein. Although several IKKε targets have been described, direct IKKε substrates necessary for regulating cell transformation have not yet been identified. Here, we performed a screen for putative IKKε substrates using an unbiased proteomic and bioinformatic approach. Using a positional scanning peptide library assay, we determined the optimal phosphorylation motif for IKKε and used bioinformatic approaches to predict IKKε substrates. Of these potential substrates, serine 418 of the tumor suppressor CYLD was identified as a likely site of IKKε phosphorylation. We confirmed that CYLD is directly phosphorylated by IKKε, and that IKKε phosphorylates serine 418 *in vivo*. Phosphorylation of CYLD at serine 418 decreases its deubiquitinase activity and is necessary for IKKε-driven transformation. Together, these observations define IKKε and CYLD as an oncogene-tumor suppressor network that participates in tumorigenesis.

INTRODUCTION

Abundant evidence supports the view that chronic inflammation contributes to cancer initiation [1-3]. Subversion of many key regulatory steps involved in immune responses occurs in both hematopoietic and epithelial cancers. For example, a large number of inflammatory mediators act as oncogenes, (IKKβ, IKKε), tumor suppressors (CYLD), or pro-survival genes in specific cell types (p65, BCL-3) [1-3].

One pathway that plays a key role in inflammation and cancer is the NF-κB pathway. Canonical, NF-κB-driven, inflammation is initiated following cellular recognition of pathogens or proinflammatory cytokines. These proinflammatory stimuli activate divergent signaling pathways, all of which ultimately converge to activate the IκB kinase (IKK) complex [4]. The core IKK complex consists of the serine/threonine kinases IKKα and IKKβ, as well as a regulatory subunit, NEMO. Upon activation by a diverse set of stimuli, this kinase complex phosphorylates the NF-κB inhibitory protein IκBα, which facilitates ubiquitination of IκBα and subsequent proteasomal degradation [4]. Degradation of IκBα permits nuclear translocation of activated NF-κB and the transcriptional activation of pro-inflammatory target genes. However, in addition to this canonical mechanism of NF-κB activation, several alternative signaling pathways also converge to activate NF-κB [5-7]. Understanding these non-canonical regulatory mechanisms will provide insights into the interplay between inflammation and cancer.

IKKε (IκB kinase ε) is one such non-canonical IKK family member that plays a critical role in the regulation of interferon signaling pathways. Both specialized membrane-bound Toll-like receptors and intracellular receptors which recognize viral

nucleic acids such as dsRNA activate this serine/threonine kinase [5, 6, 8, 9]. Activated IKKε (and the related kinase TBK1) then phosphorylates interferon response factors 3 and 7 (IRF3 and IRF7), allowing their nuclear translocation and the transcriptional upregulation of genes involved in the type I interferon response [10-13]. IKKε also phosphorylates STAT1 following activation by IFNβ [14]. The transcription factor NF-κB is also activated by IKKε, although the mechanism by which IKKε regulates the canonical NF-κB pathway is not well-understood [15, 16].

We and others have identified IKKε as a breast cancer oncogene amplified and overexpressed in over 30% of breast carcinomas and breast cancer cell lines [17-19]. Forced expression of IKKε substitutes for AKT and transforms immortal human cells, and suppression of IKKε expression in breast cancer cell lines that harbor increased copy number leads to cell death [17]. Tumorigenicity induced by either IKKε or AKT requires NF-κB activation [17]. However, the specific substrates of IKKε that are involved in cell transformation remain undefined.

Familial cylindromatosis is an autosomal dominant disease characterized by the formation of benign skin tumors, primarily on the head and neck [20]. This disease results from inheritance of a gene encoding a truncation mutant of the tumor suppressor CYLD, followed by loss of heterozygosity [21]. Decreased CYLD expression has also been identified in both hepatocellular and colon cancer cell lines and tumors [22]. Furthermore, deletion of the CYLD locus has recently been shown to be associated with NF-κB activation in multiple myeloma cell lines and patient tissues [23, 24]. CYLD is a deubiquitinating enzyme (DUB) that removes Lys63 linked ubiquitin chains and acts as a negative regulator of NF-κB signaling [25-27].

CYLD deubiquitinates several NF-κB regulators, including TRAF2, TRAF6, and NEMO [25-27] as well as BCL3, a member of the NF-κB family of transcription factors [28]. Following ubiquitination, BCL-3 translocates from the cytoplasm to the nucleus where it heterodimerizes with the NF-κB subunits p50 or p52 and activates the transcription of cyclin D, a key regulator of the G1 restriction point [29]. Similar to what is observed in kindreds afflicted with familial cylindromatosis, $CYLD^{-/-}$ mice develop benign skin tumors following exposure to UV light or TPA [28]. Other studies have shown that $CYLD^{-/-}$ mice also develop multi-organ hyperinflammation and have increased susceptibility to inflammation-induced tumors [30, 31]. Little is known, however, about how CYLD activity is regulated.

Using a recently developed peptide library approach we determined the optimal motif for phosphorylation by IKKε and then incorporated this information into a bioinformatic search for likely IKKε protein substrates [32, 33]. Many of the predicted substrates are known components of inflammatory and/or oncogenic signaling pathways, including the tumor suppressor CYLD. We show here that CYLD is a substrate of IKKε and that phosphorylation of CYLD by IKKε contributes to cell transformation.

MATERIALS AND METHODS

Antibodies, Plasmids, and Reagents. Anti-Myc (9E10), anti-CYLD, anti-RIP2, and anti-GFP were obtained from Santa Cruz Technology. Anti-Myc (rabbit) and anti-GST (mouse) were from Cell Signaling Technology. Anti-Flag (M2) and anti-HA were from Covance. Anti-IKKε and anti-actin were obtained from Sigma. Lentiviral

shRNA constructs targeting murine CYLD and GFP were obtained from the RNAi Platform of the Broad Institute of Harvard and MIT. IKKε-Tide, IKKε-Y5A, and IKKε-L8A were created and HPLC purified under contract by the Tufts University Core Facility. The IκBα (IKK substrate) peptide was obtained from Upstate. Myc-CYLD and Myc-TRAF2 were created by PCR cloning into the BamHI site of the 3XMyc (pEBB) vector. GST-IKKε was created by PCR cloning into the BamHI and NotI sites of the pEBG vector. Flag-IKKε and and Myr-Flag-IKKε were used as described previously [17]. Myc-CYLD S418A, Myc-CYLD S547A, Myc-CYLD S772A, and GST-IKKε K38A were created using a modification of the QuickChange Site-directed mutagenesis protocol (Stratagene). pBabe-CYLD WT and site-directed mutants were created by subcloning into the BamHI site of the pBabe retroviral expression vector. Myc NEMO K399R, OMNI-RIP2, HA-ubiquitin, and HA-Ub K63 only, and IκBα S32,36A ("superrepressor") were used as described previously [17, 38].

Cell Culture, Transfection, Immunoprecipitations, and Western Blotting. HEK-293T and MCF-7 cells were obtained from ATCC and were grown in DMEM containing 10% FBS. NIH-3T3 cells were obtained from ATCC and grown in DMEM containing 10% bovine calf serum. Transfection was performed by polyethylenimine. For preparation of recombinant GST-IKKε and GST-IKKε K38A, and for preparation of all immunoprecipitations used in kinase assays and Western blots, cells were lysed in 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM β-glycerophosphate, 1 mM PMSF, 1 mM sodium

orthovanadate, 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 10 nM Calyculin A. For preparation of Myc-CYLD for mass spectrometry analysis, cells were lysed in the above buffer also containing 0.25% deoxycholic acid. Immunoprecipitates for LC/MS/MS were washed in lysis buffer containing 1M NaCl prior to SDS-PAGE.

***In vitro* kinase assays.** Recombinant GST-IKKε, recombinant GST-IKKε K38A, and immunoprecipitated Myc-CYLD were generated as described above. Kinase buffer contained 50 mM Tris (pH 7.5), 12 mM $MgCl_2$, 1 mM β-glycerophosphate, 100 μM ATP, and 10 μCi γ-^{32}P-ATP/reaction. Reactions were incubated at 30ºC for 1h. Recombinant GST-IKKε and GST-IKKε K38A used in the positional scanning peptide library assay were generated as described above and the assay was performed as described previously [32, 33].

Identification of *in vivo* Phosphorylation Sites by Mass Spectrometry.

Identification:

Coomassie stained SDS-PAGE gel bands containing CYLD isolated were excised and subjected to in-gel digestion and reversed-phase microcapillary LC/MS/MS using a LTQ 2D linear ion trap (Thermo Scientific, San Jose, CA) in data-dependent acquisition and positive ion mode at a flow rate of 275nL/min using a 10 cm C_{18} microcapillary column. The column was equilibrated and peptides were loaded using 0.1% acetic acid/0.9% acetonitrile/99% water then eluted with a gradient from 5% buffer B (acetonitrile) to 38% B, followed by 95% B for washing. MS/MS spectra were searched against the reversed SwissProt protein database using Sequest

(Proteomics Browser Software, Thermo Scientific) with differential modifications for Ser, Thr and Tyr phosphorylation (+79.97) and Met oxidation (+15.99). Phosphorylation sites were identified if top ranking sequences matched to CYLD with the following Sequest scoring thresholds: 2+ ions, Xcorr ≥ 2.0, Sf ≥ 0.4, P ≥ 0; 3+ ions, Xcorr ≥ 2.75, Sf ≥ 0.5, P ≥ 0 against the forward database. Passing MS/MS spectra were then manually inspected to be sure that all b- and y- fragment ions aligned with the assigned protein database sequence. The exact sites of phosphorylation were aided using GraphMod software (PBS).

Targeted ion MS/MS:

For sites of phosphorylation in protein regions exhibiting low amino acid coverage, a targeted ion MS/MS (TIMM) experiment was performed by setting the ion trap to perform MS/MS on expected *m/z* values for predicted peptides. Ser418 (FH**pS**LPFSLTK) was not observed from a data dependent acquisition. However, it was detected via database searching from a TIMM experiment targeting the doubly charged ion at *m/z* 628.81.

NF-κB reporter assays.

MCF-7 cells: MCF-7 cells were transfected in 6-well dishes with an NF-κB-luciferase reporter (Clontech) and Renilla luciferase. Transfection efficiency was standardized using the Renilla luciferase and assays were performed according to the manufacturer's instructions (Promega).

NIH-3T3 cells: NF-κB activity was measured using the Dual-Glo Luciferase assay (Promega). IKKε-transformed NIH-3T3 cells stably expressing WT or mutant CYLD

were transiently transfected with pTRH1-NF-κB-Luciferase reporter construct in parallel to pRL-SV40-Renilla. Additional ectopic WT and mutant CYLD was also introduced in these experiments using the expression constructs MYC-CYLD, Myc-CYLD S418A, or Myc-CYLD S772A. Luciferase values were normalized to Renilla values to calculate relative light units (RLU).

***In vitro* cell transformation assays.**

Growth of NIH-3T3 cells in soft agar was determined by plating 5 X 10^4 cells in triplicate in 0.4% Noble agar and DMEM containing 10% bovine calf serum. Colonies greater than 100 μm in diameter were counted microscopically 21 days after plating.

Tumorigenicity assay.

A total of 2 X 10^6 cells were subcutaneously injected into immunodeficient mice (Balb/c Nude, Charles River Laboratories) anesthetized with isofluorane. Two mice were used per group, and three injection sites were made per mouse. Tumors were measured at 21 days post-injection.

IKKε phospho-substrate antibody.

Rabbits were immunized with a phosphopeptide library with fixed residues corresponding to the IKKε phosphorylation motif and antibodies were purified as has been described previously [40].

RESULTS

Determination of IKKε substrate specificity

IKKε has recently been identified as a breast cancer oncogene. However, the mechanism by which IKKε contributes to cell transformation is not known. Since IKKε is a serine/threonine kinase, we sought to determine whether the kinase activity of IKKε is necessary for its oncogenic activity. Wild-type IKKε or kinase-dead IKKε K38A was introduced into NIH-3T3 cells (Figure 3.1A). We found that cells expressing WT IKKε, but not IKKε K38A, exhibited robust anchorage-independent colony growth (Figure 3.1B). These results confirm that the kinase activity of IKKε is necessary for its activity as an oncogene.

While several IKKε substrates involved in interferon signaling pathways have been identified, direct substrates required for the oncogenic functions of IKKε have not been reported. To address this question, we utilized proteomic and bioinformatic approaches to perform an unbiased screen for likely IKKε substrates. Specifically, we used a positional scanning peptide library assay recently developed in this laboratory to identify the optimal phosphorylation motif for IKKε [32, 33]. This assay employs 198 biotinylated peptide libraries. Each library has a 50-50 mixture of serine and threonine fixed at the central position, and has one additional position fixed to one of the 20 naturally-occurring amino acids. All other positions contain a

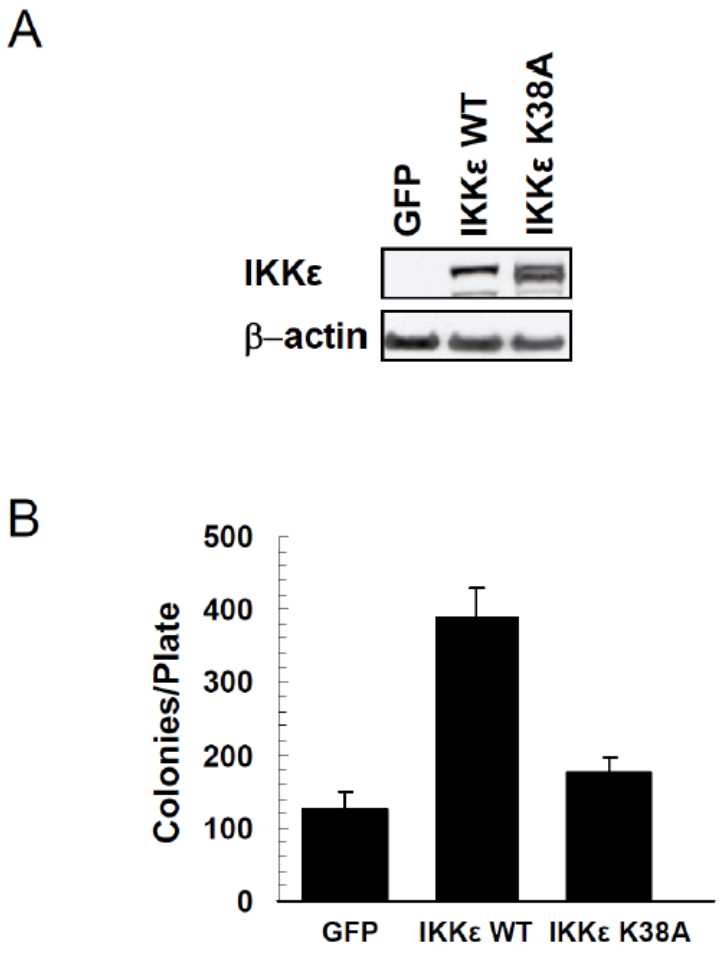

Figure 3.1. IKKε mediates cell transformation
(A) NIH-3T3 cells were stably transduced with Flag-IKKε, kinase-dead Flag-IKKε K38A, or GFP control via lentiviral infection. Immunoblot analysis shows similar IKKε expression. F-IKKε = Flag-IKKε. (B) Flag-IKKε or Flag-IKKε K38A-expressing NIH-3T3 cells generated in (A) were assayed for anchorage-independent growth. Colony formation was examined after 21 days. Expression of wild-type IKKε, but not IKKε K38A, induces anchorage-independent growth. Error bars represent standard deviation (SD) for 3 independent experiments.

degenerate mixture of amino acids (excluding serine, threonine, and cysteine). Phosphothreonine and Phosphotyrosine were included at the fixed positions in order to facilitate the identification of kinases which have a requirement for priming phosphorylation events. Using recombinant GST-IKKε purified from HEK-293T cells, we performed kinase assays simultaneously on all 198 peptide libraries in solution using γ-^{32}P-ATP. Biotinylated peptides were captured with a streptavidin-coated membrane and the relative preference for each amino acid at each position was determined by the relative level of radiolabel incorporation into the corresponding peptides.

We found that IKKε exhibits strong sequence selectivity at multiple positions surrounding the phosphorylation site. IKKε strongly prefers substrates with a hydrophobic residue at the +1 position relative to the phosphorylation site (Figures 3.2A and 3.2E). This kinase also exhibits strong sequence selectivity for aromatic residues at the -2 position, and for bulky hydrophobic residues at the +3 position (Figures 3.2A and 3.2E). A kinase-dead IKKε K38A does not exhibit selectivity at these positions, confirming that peptides were not being phosphorylated by a contaminating kinase (Figure 3.2B).

To validate this motif, we generated a consensus peptide substrate, IKKε-Tide, and measured the phosphorylation of peptides bearing individual alanine substitutions. By comparing these results to those obtained using the consensus sequence we confirmed that substitution of amino acids at the +1 or -2 positions resulted in a decrease in the efficiency of peptide phosphorylation (Figure 3.2C). This motif has similarities to the sequence surrounding the autophosphorylation site

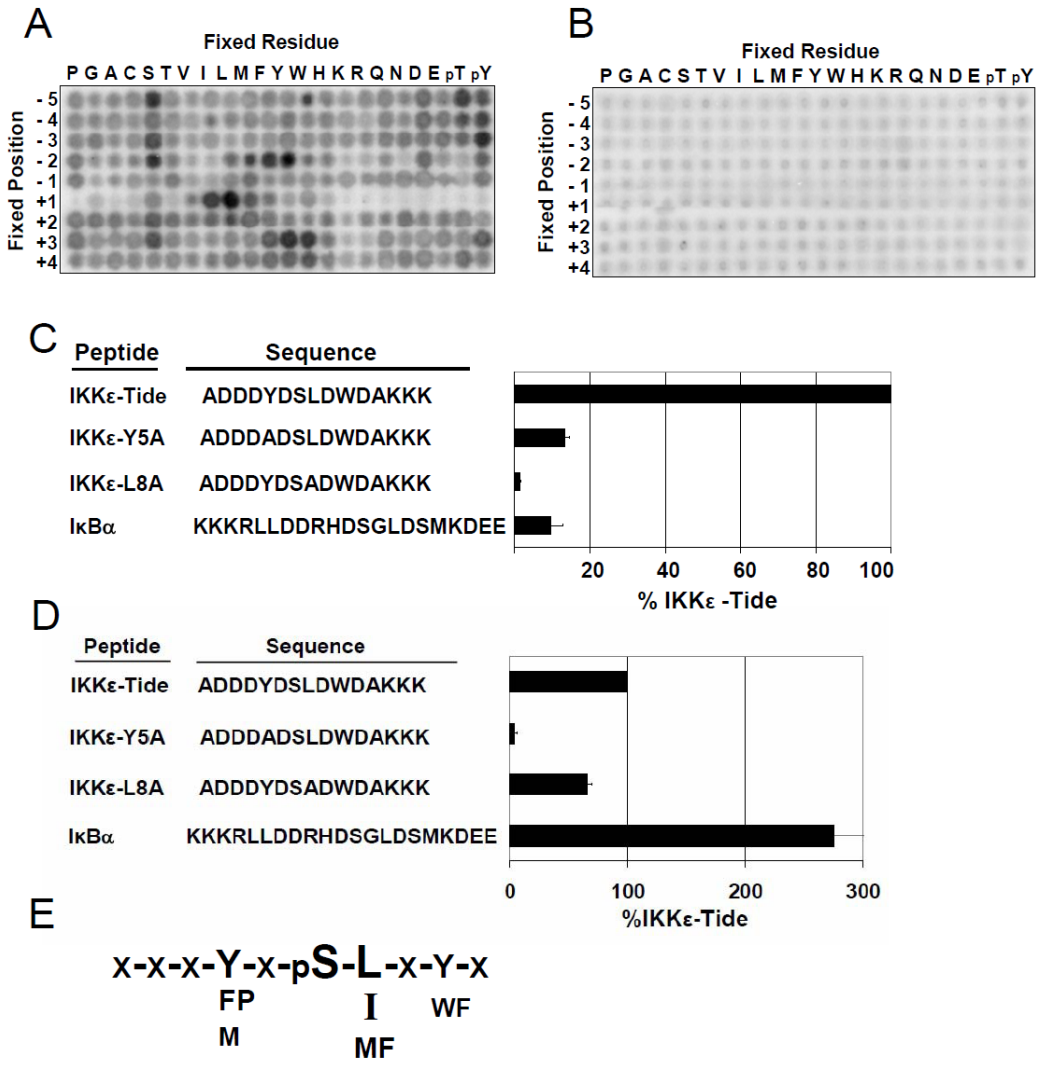

Figure 3.2: Identification of the optimal IKKε phosphorylation motif
(A-B) Recombinant IKKε or kinase-dead IKKε K38A was used to phosphorylate 198 peptide libraries in individual kinase assays. The general sequence for these libraries is Y-A-X-X-X-Z-X-S/T-X-X-X-X-A-G-K-K-biotin (Z = fixed amino acid, X = equimolar mixture of amino acids excluding Ser, Thr, and Cys). After binding to a streptavidin-coated membrane, phosphorylation was visualized by the incorporation of ^{32}P. WT IKKε (A) gave a strong, consistent motif. No motif could be identified using IKKε K38A (B). (C and D) Individual peptides were phosphorylated with recombinant IKKε(C) and IKKβ(D). Phosphorylation is shown as a percentage of the rate of phosphorylation of IKKε-Tide, the optimal peptide substrate for IKKε as determined in (A). Alteration of critical amino acids to alanine decreased peptide phosphorylation. A peptide corresponding to the sequence surrounding Ser32 and Ser36 of IκBα was not efficiently phosphorylated by IKKε. Error bars depict SD. (E) Primary and secondary selections determined from IKKε phosphorylation motif.

in the activation loop of IKKε (DEKFV**S**$_{172}$VYGTE) due to the aromatic residue at -2 and aliphatic at +1, although this site is predicted to be suboptimal due to the lack of an aromatic residue at +3 [15].

IKKε has been shown to phosphorylate one of the essential serines on IκBα *in vitro*, but *in vivo* phosphorylation of IκBα by this kinase has not been described [15]. Therefore, the role of IKKε in IκBα phosphorylation and degradation remains unclear. To examine this question, we performed kinase assays using a peptide substrate corresponding to the sequence surrounding Ser32 and Ser36 of IκBα. This peptide contains two potential phosphorylation sites, but neither site is within a sequence context that matches the optimal motif for IKKε. We found that this peptide was a poor *in vitro* substrate for IKKε when compared with the optimal peptide determined from the peptide library screen (Figure 3.2E). In contrast, when recombinant GST-IKKβ was used to phosphorylate the same set of peptides, the IκBα peptide was phosphorylated by IKKβ substantially more efficiently than IKKε-Tide (Figure 3.2D). These observations suggest that IκBα is unlikely to be an important physiological substrate of IKKε. We recently demonstrated that, like IKKε, IKKβ prefers aromatic residues at the -2 position and hydrophobic residues at the +1 position [34]. However, the phosphorylation motifs for these kinases differ at the -4, -5, and +3 positions. Taken together, these observations demonstrate that while the substrate specificities of IKKβ and the related kinase IKKε have overlapping characteristics, the optimal substrate peptides for these kinases differ in substantial ways and therefore be predicted to have different (though possibly overlapping) *in vivo* substrates.

Prediction of IKKε substrates

Spot intensities from the peptide library screen were then quantified and converted into a matrix which could be used with the bioinformatic search engine Scansite (Table 3.1). Scansite (http://scansite.mit.edu) allows proteome-wide searches for sites which best match the data provided by the input matrix [35, 36]. Table 3.2 shows top-scoring candidate IKKε substrates obtained following the Scansite analysis. All of these potential phosphorylation sites scored in the top 0.05% of sites in the SwissProt database. Interestingly, a large number of predicted IKKε substrates are known to be involved in inflammatory and/or oncogenic signaling pathways. Of these potential substrates, the deubiquitinating enzyme CYLD was of particular interest, as it has been shown to have roles as both an inflammatory mediator and tumor suppressor, functions that could be downstream of IKKε [21]. Our bioinformatic analysis predicted that CYLD is likely to be phosphorylated by IKKε at Ser418.

CYLD is phosphorylated by IKKε at Ser418

To further facilitate the identification of novel IKKε substrates we raised antibodies against a collection of phosphopeptides biased towards the optimal IKKε phosphorylation motif.We verified that these antibodies recognize known IKKε substrates in a kinase-dependent manner (data not shown). These antibodies were then used to determine whether CYLD is phosphorylated at a site matching the IKKε phosphorylation motif. HEK-293T cells were cotransfected with Myc-epitope tagged

Table 3.1. **IKKε specificity values**

Amino Acid	Position								
	-5	-4	-3	-2	-1	+1	+2	+3	+4
P	0.95	1.15	0.94	1.08	1.30	0.11	0.80	1.15	0.90
G	0.73	1.08	1.24	0.57	0.42	0.49	0.90	0.73	0.81
A	0.93	1.04	0.77	0.63	0.71	0.22	0.88	0.61	1.02
C	0.97	0.79	0.76	0.67	0.87	0.42	0.60	0.78	0.89
S	3.22	1.85	2.41	2.41	1.01	1.31	1.59	1.95	0.92
T	0.78	0.90	0.87	0.84	1.44	0.47	1.01	0.84	0.91
V	0.48	0.43	0.48	0.31	0.62	1.15	0.75	0.51	0.79
I	0.63	1.07	0.79	0.25	0.57	3.44	1.25	0.62	0.90
L	0.45	0.99	0.47	1.23	1.12	5.30	1.68	0.79	1.37
M	0.56	0.99	0.76	1.63	1.77	2.29	1.66	0.70	1.13
F	0.95	0.78	0.77	2.33	0.96	1.44	1.07	1.65	1.48
Y	0.76	1.40	1.22	3.14	1.12	0.70	0.74	3.05	1.73
W	1.77	0.87	0.79	0.98	1.00	1.08	0.70	2.50	2.00
H	0.75	1.04	0.92	0.77	0.96	0.48	1.00	0.98	0.90
K	0.46	0.49	0.79	0.16	0.80	0.14	0.30	0.17	0.39
R	0.78	0.22	0.80	0.25	0.82	0.15	0.78	0.20	0.46
Q	1.01	0.95	1.13	0.60	1.13	0.20	0.89	0.72	0.74
N	0.87	0.72	1.27	0.21	1.29	0.22	1.38	0.62	1.03
D	1.87	1.86	1.38	1.29	1.18	0.25	0.93	0.60	0.92
E	1.08	1.37	1.46	0.63	0.91	0.12	1.10	0.83	0.71

Following the positional scanning peptide library assay, phosphate incorporation for each amino acid at each position was quantified using the ImageQuant 5.2 software from Molecular Dynamics. The phosphate incorporation for each amino acid was then divided by the average phosphate incorporation for the amino acids in that row. These values represent the average of two independent assays.

Table 3.2. Candidate IKKε substrates

Symbol	Name	Site	Sequence
CYLD_HUMAN	Deubiquitinating enzyme CYLD	418	ENRFH**S**LPFSL
NED4_HUMAN	E3 ubiquitin-protein ligase Nedd-4	784	SEYYN**S**LRWIL
MM21_HUMAN	Matrix metalloproteinase-21 precursor (MMP-21)	517	SYAYN**S**IFFFK
MYCN_HUMAN	N-myc proto-oncogene protein	23	DLEFD**S**LQPCF
ICE8_HUMAN	Caspase-8 precursor	26	SLKFL**S**LDYIP
GNRP_HUMAN	Guanine nucleotide releasing protein (Ras-specific nucleotide exchange factor CDC25)	667	DLRFL**S**IDFLN
TLR4_HUMAN	Toll-like receptor 4 precursor	100	DGAYQ**S**LSHLS
ZN20_HUMAN	Zinc finger protein 20 (Zinc finger protein KOX13)	297	FISFS**S**IQYHK
SGK3_HUMAN	Serine/threonine-protein kinase Sgk3	421	HPFFE**S**LSWAD
MTA3_HUMAN	Metastasis associated protein MTA3	150	DFFFY**S**LVYDP
DZP3_HUMAN	Ubiquitin ligase protein DZIP3	179	CENFM**S**LVYFG
TANK_HUMAN	TRAF family member-associated NF-kappa-B activator (I-TRAF)	354	DAPFP**S**LDSPG
UP2L_HUMAN	Ubiquitin associated protein 2-like	910	GYSYT**S**LPYYT
TF65_HUMAN	Transcription factor p65 (Nuclear factor NF-kappa-B p65 subunit)	536	DEDFS**S**IADMD
TLR2_HUMAN	Toll-like receptor 2 precursor	98	EDSFS**S**LGSLE
ZB24_HUMAN	Zinc finger and BTB domain containing protein 24 (Zinc finger protein 450)	63	SSEYF**S**MMFAE
I12B_HUMAN	Interleukin-12 beta chain precursor (IL-12B)	270	PHSYF**S**LTFCV
GABJ_HUMAN	GRB2-associated binding protein 2	488	AHHFD**S**LGYPS
TLR8_HUMAN	Toll-like receptor 8 precursor	212	SLSFN**S**LSHVP
NAL7_HUMAN	NACHT-, LRR- and PYD-containing protein 7	27	LKSFK**S**LLWAF
BAR1_HUMAN	BRCA1-associated RING domain protein 1 (BARD-1)	389	SDEFI**S**LSPGT
KG88_HUMAN	Protein KIAA1688	384	PERFL**S**LEYSP
PPE2_HUMAN	Serine/threonine protein phosphatase with EF-hands-2 (PPEF-2)	50	WSIFQ**S**IEYAG
PMS1_HUMAN	PMS1 protein homolog 1 (DNA mismatch repair protein PMS1)	251	DHSFT**S**LSTPE
P2BB_HUMAN	Serine/threonine protein phosphatase 2B catalytic subunit, beta isoform	506	QDGFN**S**LNTAH
DD15_HUMAN	Putative pre-mRNA splicing factor RNA helicase (DEAH box protein 15)	653	FINYR**S**LMSAD

A Scansite matrix was created from IKKε specificity values (Table 3.1). Scansite (scansite.mit.edu) was then used to search the SwissProt database for sites which correspond to the IKKε phosphorylation motif. Included sites scored in the top 0.05% of sites searched.

CYLD (Myc-CYLD) and either GST-IKKε WT or kinase-dead IKKε K38A. CYLD was immunoprecipitated via its Myc tag and immunoblotted with the anti-IKKε phospho-substrate antibody. Figure 3.3A shows that the phospho-substrate antibody blotted CYLD which had been transfected with WT IKKε, but not IKKε K38A. CYLD treated with calf-intestinal phosphatase (CIP) following cotransfection with IKKε was no longer recognized by the phospho-substrate antibody, confirming that the IKKε phospho-substrate antibody specifically recognizes phosphorylated CYLD (Figure 3.3B).

In order to determine whether IKKε can directly phosphorylate CYLD, an *in vitro* kinase assay was performed. Wild-type GST-IKKε or GST-IKKε K38A was purified from HEK-293T cells. Myc-CYLD was separately transfected into HEK-293T cells and immunoprecipitated. When the CYLD immunoprecipitate was incubated in an *in vitro* kinase assay with WT IKKε, strong phosphorylation of CYLD was observed (Figure 3.3C). This phosphorylation was not observed in the presence of IKKε K38A.

To determine whether IKKε and CYLD physically interact, Myc-CYLD was cotransfected into HEK-293T cells expressing GST- IKKε WT or K38A. CYLD was immunoprecipitated via its Myc tag and these immune complexes were blotted with an anti-GST antibody to identify IKKε. In CYLD immune complexes we identified both WT and kinase-dead IKKε (Figure 3.4A). Moreover, when we performed the reciprocal experiment, we found that Myc-CYLD was also observed in the IKKε precipitates (Figure 3.4B).

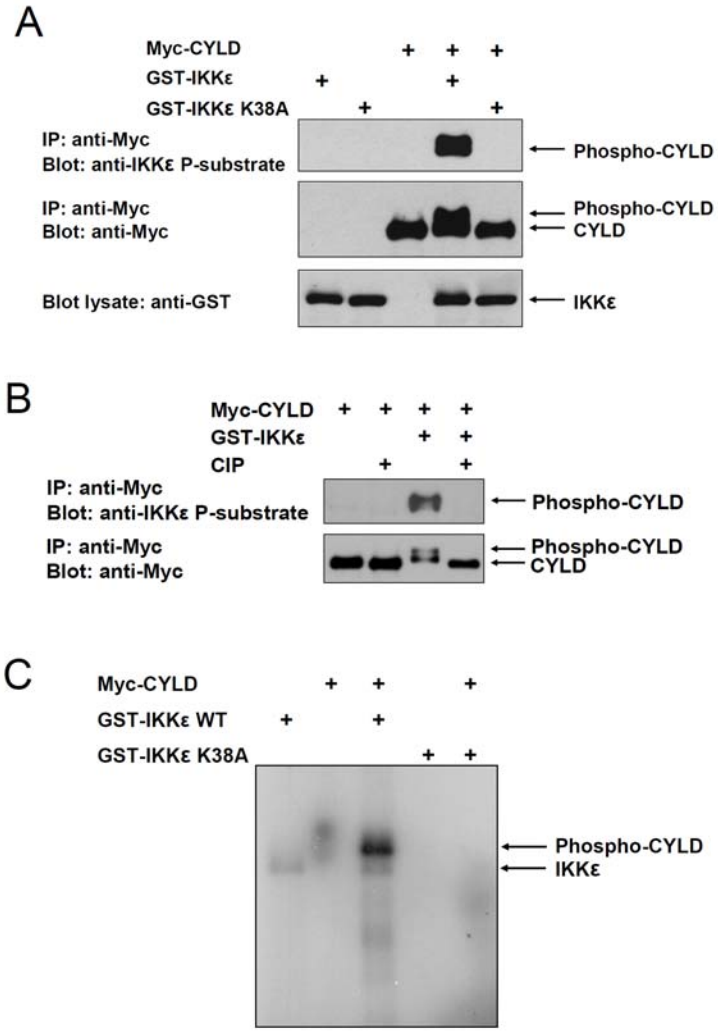

Figure 3.3. IKKε phosphorylates CYLD at a site corresponding to the IKKε phosphorylation motif

(A) Myc-CYLD was cotransfected with GST-IKKε WT or K38A into HEK-293T cells. Myc-CYLD was immunoprecipitated and immune complexes were immunoblotted with an IKKε phospho-substrate antibody. CYLD was recognized by the phospho-substrate antibody when cotransfected with WT IKKε, but not kinase-dead IKKε. (B) Myc-CYLD was transfected into HEK-293T cells alone or with GST-IKKε. CYLD was immunoprecipitated with an anti-Myc antibody. Immunoprecipitates were divided into two samples and treated with either Calf-intestinal phosphatase (CIP) or water (as a control) for 45 min at 37ºC. Samples were then blotted with an IKKε phospho-substrate antibody (C) Recombinant GST-IKKε or IKKε K38A was purified from HEK-293T cells. Myc-CYLD was transfected separately into HEK-293T cells and immunoprecipitated. CYLD immune complexes were incubated in the presence of γ-^{32}P-ATP with either WT or kinase-dead IKKε.

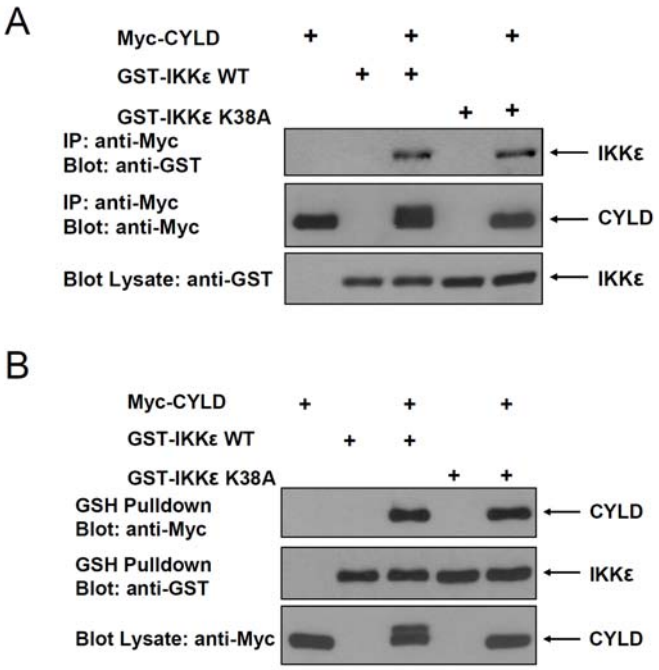

Figure 3.4. CYLD physically interacts with IKKε

Myc-CYLD was cotransfected into HEK-293T cells with GST-IKKε WT or GST-IKKε K38A. (A) Lysates were subjected to immunoprecipitation with an anti-Myc antibody and immunoprecipitates were blotted with an anti-GST antibody. (B) Lysates were subjected to precipitation with glutathione-conjugated Sepharose beads and precipitates were blotted with an anti-Myc antibody. CYLD interacts with both WT and kinase-dead IKKε.

While CYLD Ser418 was predicted by Scansite to be the optimal site for IKKε phosphorylation (ENRFH**S**$_{418}$LPFSL), two additional serines within the CYLD sequence were potential, though less optimal, IKKε phosphorylation sites (DSRFA**S**$_{547}$LQPVS and KKIFP**S**$_{772}$LELNI). Therefore, we used mass spectrometry to determine which residue(s) of CYLD is phosphorylated *in vivo*. Myc-CYLD and GST-IKKε were cotransfected into HEK-293T cells. CYLD was immunoprecipitated, subjected to SDS-PAGE, and Coomassie stained (Figure 3.5A). The band corresponding to Myc-CYLD was excised, digested with trypsin or chymotrypsin, and subjected to microcapillary LC/MS/MS. A phosphopeptide consistent with phosphorylation at Ser418 was identified. This analysis confirmed that CYLD is phosphorylated at Ser418, the same site predicted by our bioinformatic analysis (Figure 3.5B). Ser418 is evolutionarily conserved in all sequenced mammals, as well as *Gallus gallus* and *Xenopus tropicalis* (Figure 3.5C). In addition, the -2F, +1L, and +3F relative to Ser418 (which correspond to the IKKε phosphorylation motif) are also conserved, providing further evidence for the evolutionary importance of this phosphorylation site.

To further verify that Ser418 is the critical site phosphorylated by IKKε, we created Myc-epitope tagged mutants of CYLD at Ser418 (S418A), Ser547 (S547A), and Ser772 (S772A). We introduced wild-type CYLD or each of these mutants into HEK-293T cells alone or with GST-IKKε and isolated CYLD immune complexes. Mutation of either Ser547 or Ser772 did not affect recognition of CYLD by the IKKε phospho-substrate antibody (Figure 3.6A). However, Myc-CYLD S418A was no longer recognized by the IKKε phospho-substrate antibody when cotransfected with

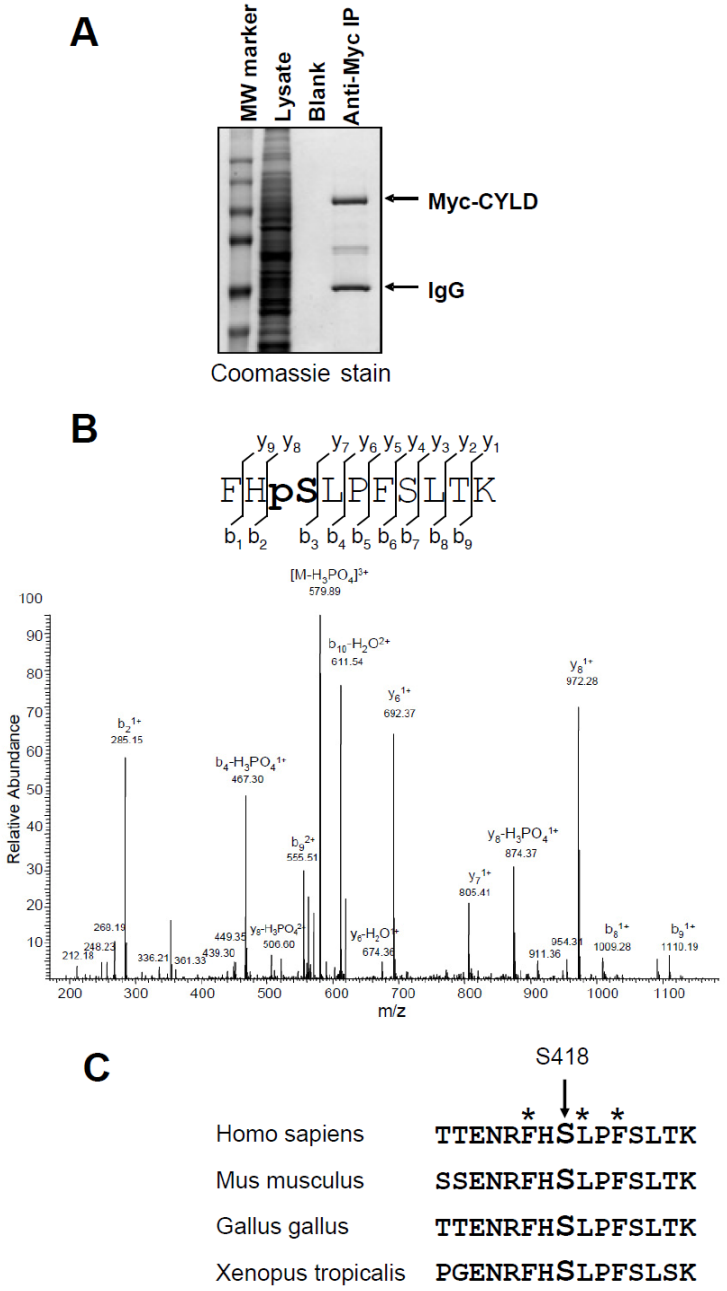

Figure 3.5 Identification of CYLD phosphorylation site
(A) Myc-CYLD was cotransfected into HEK-293T cells with GST-IKKε. Myc-CYLD was immunoprecipitated and the immunoprecipitate was subjected to SDS-PAGE followed by Coomassie staining. (B)The band corresponding to CYLD was excised from the gel, and digested with trypsin and chymotrypsin. Phosphorylation sites were mapped by microcapillary LC/MS/MS, resulting in 85% coverage of the CYLD amino acid sequence. A phosphopeptide consistent with phosphorylation at Ser418 was identified. (C) Ser418 of CYLD and surrounding residues are evolutionarily conserved.

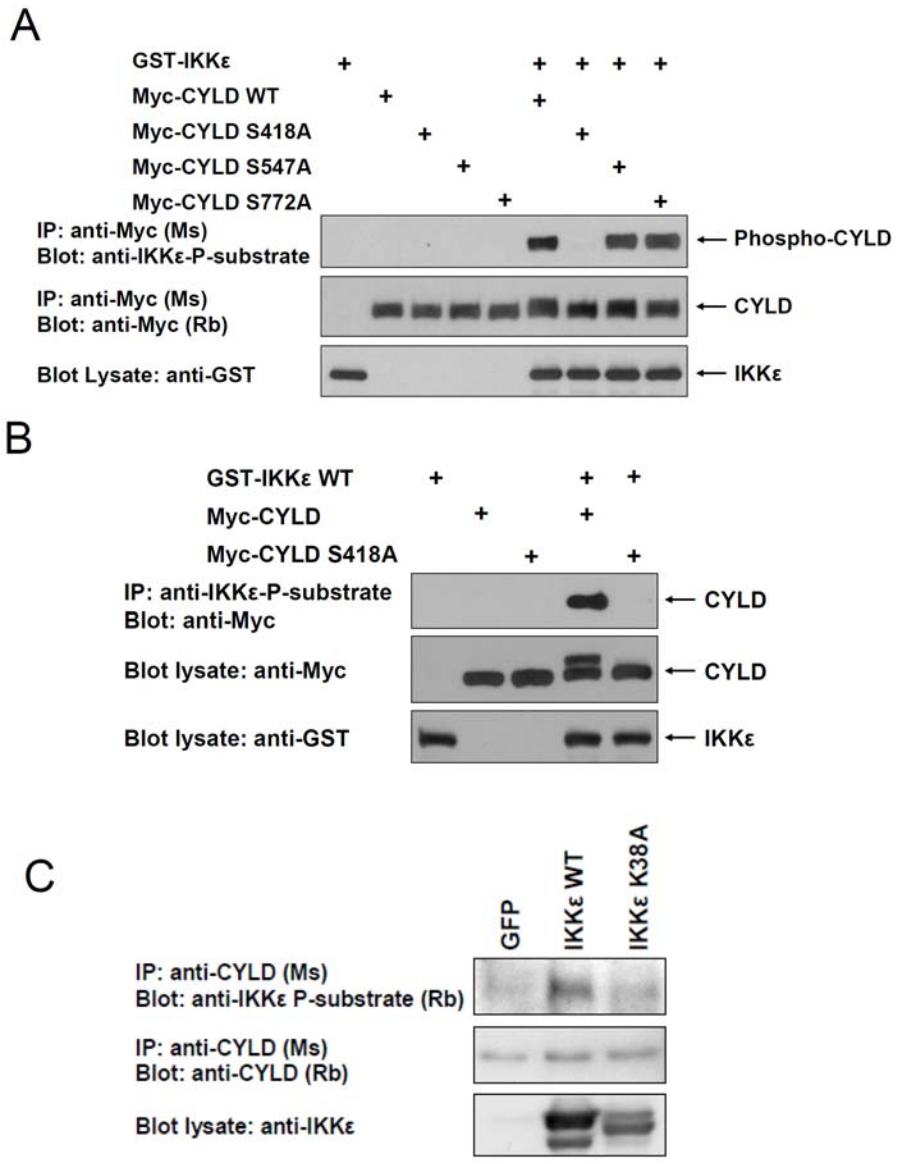

Figure 3.6. CYLD is phosphorylated by IKKε at Ser418.
(A) Cell were transfected as indicated. Lysates were subjected to immunoprecipitation with an anti-Myc antibody and immunoprecipitates were blotted with an anti-IKKε phospho-substrate antibody. (B) Myc-CYLD WT or Myc-CYLD S418A was cotransfected into HEK-293T cells with GST- IKKε. Lysates were subjected to immunoprecipitation with an IKKε phospho-substrate antibody. Immunoprecipitates were blotted with an anti-Myc antibody. (C) Endogenous CYLD was immunoprecipitated from IKKε-transformed NIH-3T3 cells generated in Figure 3.1A. Immunoprecipitates were blotted with an IKKε-phospho-substrate antibody.

IKKε (Figure 3.6A). In addition, when we introduced Myc-CYLD or Myc-CYLD S418A with IKKε into HEK-293T cells and performed an immunoprecipitation using the IKKε phospho-substrate antibody, we found that wild-type Myc-CYLD, but not Myc-CYLD S418A, was efficiently immunoprecipitated by the IKKε phospho-substrate antibody (Figure 3.6B). Finally, endogenous CYLD was immunoprecipitated from IKKε-transformed NIH-3T3 cells utilized in Figure 3.1. Immunoblotting with the IKKε phospho-substrate antibody revealed that CYLD is indeed phosphorylated in cells transformed by IKKε, but not in cells expressing IKKε K38A (Figure 3.6C). Together, these data confirm that CYLD Ser418 is phosphorylated *in vivo* in the presence of IKKε.

It has been reported that Ser418 of CYLD is a substrate of the canonical IKK family members, IKKβ and IKKα [37]. Therefore, in order to determine which IKK family member(s) can most efficiently phosphorylate CYLD, we cotransfected Myc-CYLD with individual IKK family members fused to GST. CYLD was immunoprecipitated using an anti-Myc antibody and blotted using the anti-IKKε phospho-substrate antibody. Figure 3.7 shows that CYLD was efficiently phosphorylated in cells transfected with IKKε. In contrast, CYLD is phosphorylated inefficiently in cells transfected with IKKα and IKKβ, even when greater than 5X more kinase is present (Figure 3.7). While it is therefore possible for any of these IKK family members to phosphorylate Ser418 of CYLD, CYLD is a much better substrate for IKKε than for IKKα or IKKβ. Our analysis of IKKε and IKKβ peptide substrate specificities also demonstrated that these kinases should be expected to have

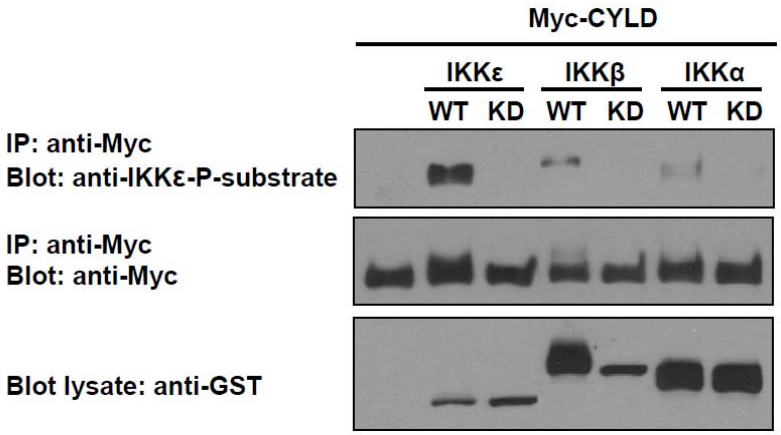

Figure 3.7. Phosphorylation of CYLD by IKK family members.
Myc-CYLD was cotransfected into HEK-293T cells with IKKα, IKKβ, or IKKε (WT or kinase-dead). CYLD was immunoprecipitated via its Myc tag, and immunoprecipitates were blotted with an anti-IKKε phospho-substrate antibody. KD = Kinase-dead

largely different, though possibly overlapping, substrate pools (Figures 3.2C and 3.2D).

IKKε-mediated phosphorylation of CYLD at S418 facilitates transformation

Since CYLD is a known tumor suppressor and IKKε is a newly-discovered oncoprotein [17, 21, 28], we hypothesized that phosphorylation of CYLD by IKKε might play a role in the regulation of IKKε-mediated cell transformation. In Figure 3.1 we show that forced expression of IKKε induces a tumorigenic phenotype in NIH-3T3 cells. To determine whether suppression of CYLD also induced cell transformation in this experimental model, we suppressed murine CYLD using two distinct short hairpin RNAs (shRNA), each of which strongly suppresses endogenous levels of CYLD (Figure 3.8A). Suppression of CYLD induced substantial anchorage-independent growth of NIH-3T3 cells when compared to control NIH-3T3 cells (Figures 3.8B and 3.8C).

To determine whether CYLD phosphorylation is required for IKKε-mediated transformation, we generated IKKε-transformed NIH-3T3 cells that stably express wild-type CYLD, CYLD S418A, or CYLD S772A (Figure 3.9A). We found that Flag-epitope tagged IKKε-expressing cells exhibited robust anchorage-independent growth that was 4-fold above that observed in control NIH-3T3 cells or cells expressing WT CYLD or CYLD S772A (Figure 3.9B). In contrast, in tumorigenic cells expressing Flag-epitope-tagged IKKε, co-expression of CYLD S418A suppressed anchorage-independent growth (Figure 3.9B). A similar result was observed *in vivo* when we evaluated the contribution of CYLD phosphorylation to

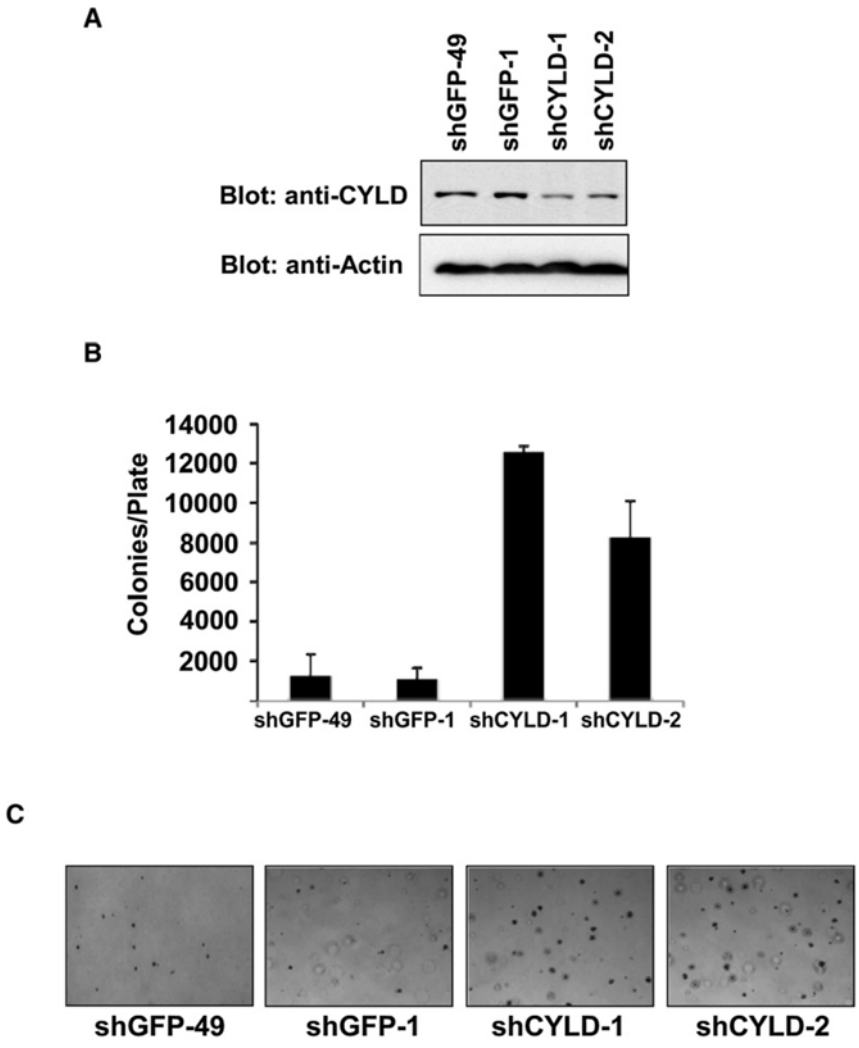

Figure 3.8. Suppression of CYLD protein expression is sufficient to induce transformation.
(A) Two distinct shRNA constructs targeting murine CYLD (shCYLD1 and shCYLD2) were transduced into NIH 3T3 cells via lentiviral infection. Two shRNAs targeting GFP (shGFP1 and shGFP49) were used as controls. Both shCYLD1 and shCYLD2 induce substantial knockdown of endogenous CYLD.
(B and C) Anchorage-independent growth of NIH 3T3 cells was assessed following suppression of endogenous CYLD with shCYLD1 or shCYLD2 for 21 days. (B) Colony number and (C) Colony formation (at 103 magnification) were examined. Error bars depict SD for three independent experiments.

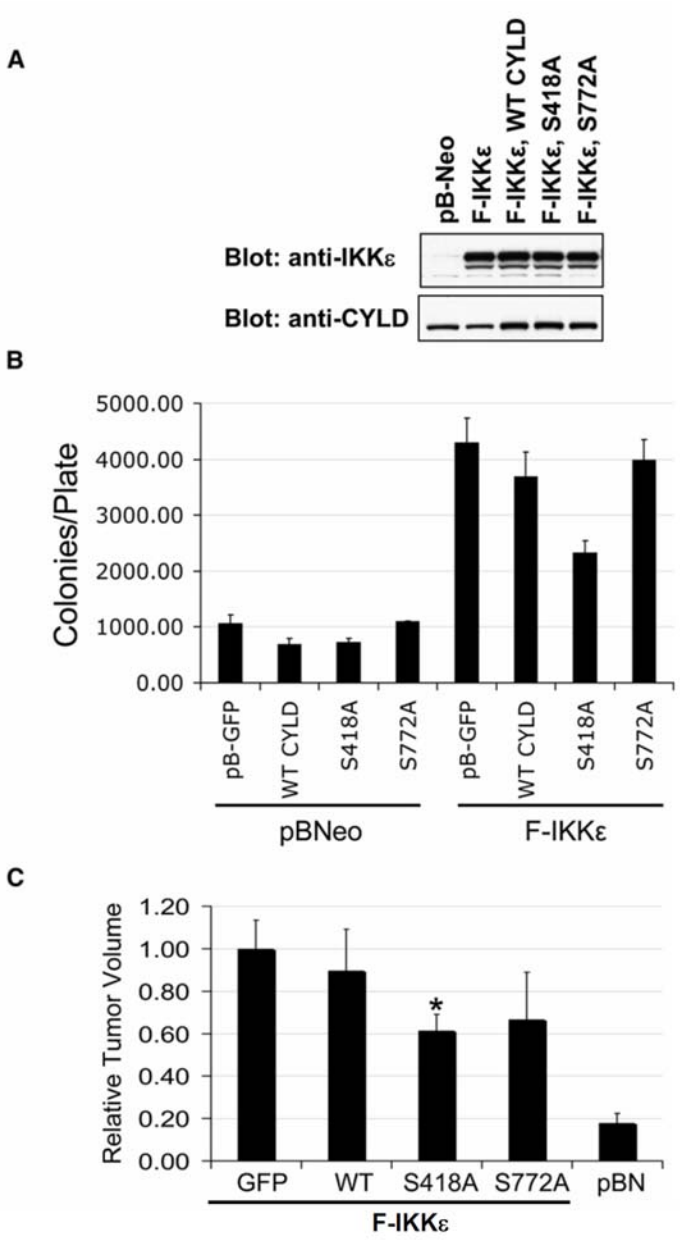

Figure 3.9. CYLD phosphorylation at serine 418 is important for IKKε-mediated transformation.

(A) IKKε-transformed NIH-3T3 cells were stably transduced with WT-CYLD, CYLD S418A, CYLD S772A, or vector control. Immunoblot analysis for CYLD and IKKε were performed. (B) Flag-IKKε transformed NIH-3T3 cells expressing WT CYLD or CYLD mutants generated in (A) were assayed for anchorage-independent growth. Colony formation was examined after 21 days. (C) IKKε-transformed NIH-3T3 cells expressing WT CYLD, CYLD S418A, CYLD S772A, or GFP control were subcutaneously introduced into immunodeficient mice. Relative tumor volume was assessed at 21 days post injection. Astericks indicates statistical significance as determined by a student's t-test ($p \leq 0.05$).

IKKε-induced tumorigenicity. Introduction of IKKε-transformed NIH-3T3 cells into immunodeficient animals yielded tumor formation at a high penetrance (Figure 3.9C, data not shown). While expression of WT CYLD or CYLD S772A failed to significantly alter tumorigenicity, expression of CYLD S418A led to statistically-significantly smaller tumors ($p<0.05$) (Figure 3.9C). Taken together, these findings demonstrate that phosphorylation of CYLD by IKKε at serine 418 is necessary for IKKε to fully induce transformation.

Phosphorylation of CYLD at Ser418 decreases CYLD activity

CYLD is a deubiquitinating enzyme which can remove Lys63 linked ubiquitin chains from a large number of proteins, including the inflammatory mediators TRAF2, TRAF6, and NEMO, as well as the pro-proliferation transcription factor BCL-3 [25-28]. Removal of these ubiquitin chains by CYLD inactivates these substrates, downregulating inflammatory signaling and progression through the cell cycle. Upon loss of CYLD protein level or activity, therefore, cell transformation is hypothesized to occur following the accumulation of a variety of ubiquitinated species, which leads to both increased cell proliferation and the increased transcription of NF-κB-regulated anti-apoptotic factors. Therefore, we sought to determine the effect of CYLD phosphorylation on ubiquitination of known CYLD substrates.

TRAF2 is a critical NF-κB mediator ubiquitinated via Lys63-linked ubiquitin chains following stimulation with TNFα, and CYLD removes these ubiquitin chains to prevent uncontrolled TNFα-induced inflammation. To determine the effect of

CYLD phosphorylation on TRAF2 ubiquitination, HEK-293T cells were transfected with Myc-epitope tagged TRAF2 in combination with GFP-WT CYLD or CYLD S418A and GST-IKKε. In order to examine only changes in Lys63 linked ubiquitin chains, cells were also transfected with an HA-tagged ubiquitin construct which has all lysines mutated to arginine except for Lys63 (HA-Ub-K63). Cells were stimulated with TNFα for 10 minutes prior to lysis. Lysates were immunoprecipitated with an anti-Myc antibody and blotted with an anti-HA antibody. As expected, CYLD efficiently removed Lys63-linked ubiquitin chains from TRAF2 (Figure 3.10A). Interestingly, coexpression of IKKε blocked CYLD-mediated deubiquitination of TRAF2, demonstrating that phosphorylated CYLD exhibited less deubiquitinating activity than unphosphorylated CYLD. In contrast, cotransfection of CYLD S418A with IKKε did not block deubiquitination of TRAF2 (Figure 3.10A). Thus, IKKε regulates the deubiquitination of TRAF2 by CYLD.

The IKK regulatory subunit NEMO also undergoes Lys63-specific ubiquitination at Lys285 when coexpressed with RIP2, resulting in increased NF-κB activity [38, 39]. CYLD efficiently removes these RIP2-induced ubiquitin chains from NEMO [38]. To determine whether phosphorylation of CYLD also affects RIP2-induced NEMO ubiquitination, HEK-293T cells were cotransfected with Myc-NEMO, OMNI-RIP2, HA-Ubiquitin, GFP-CYLD, and GST-IKKε. As expected, cotransfection of RIP2 with NEMO increased NEMO ubiquitination, and this ubiquitination was suppressed by CYLD (Figure 3.10B). However, cotransfection of IKKε with CYLD resulted in increased NEMO ubiquitination, suggesting that

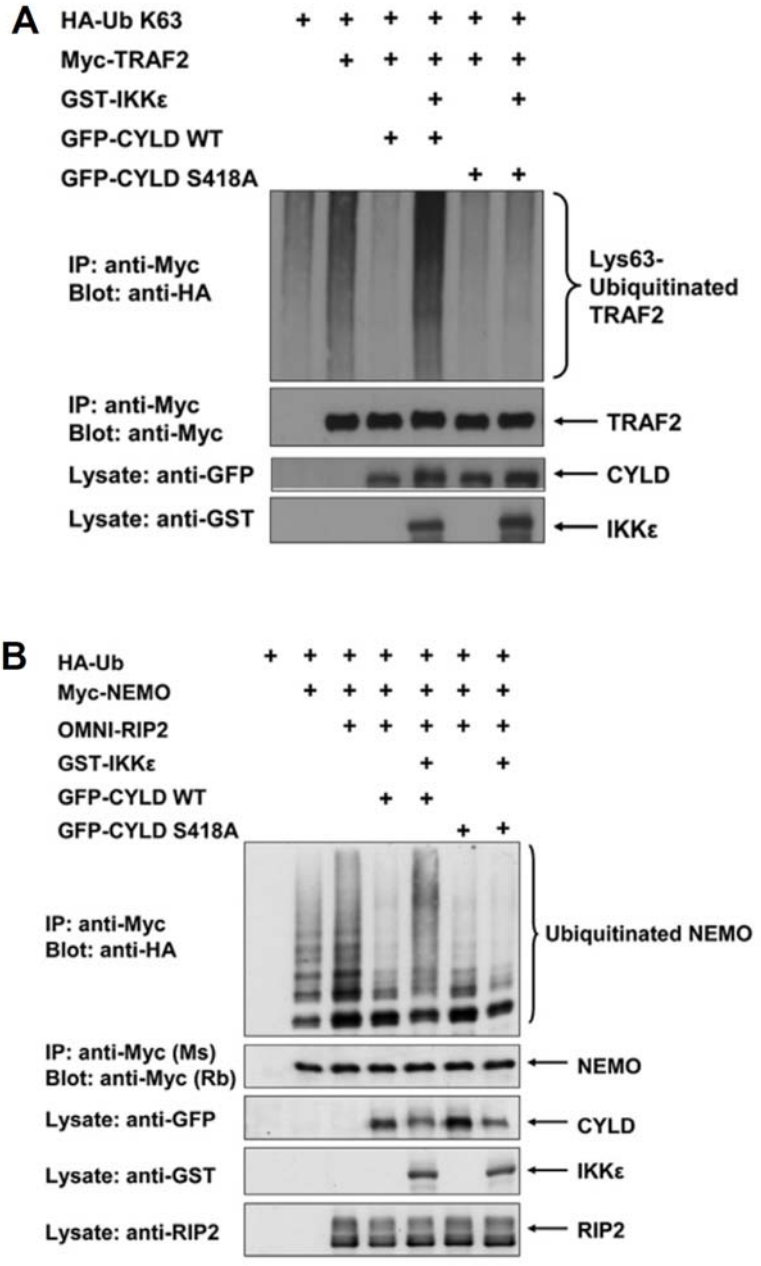

Figure 3.10. Phosphorylation of CYLD at Ser418 decreases CYLD DUB activity (A) HEK-293T cells were transfected with HA-Ub K63, Myc-TRAF2, GFP-CYLD WT or S418A, and GST-IKKε. Cells were stimulated with 10 ng/mL TNFα for 10 minutes prior to lysis. TRAF2 was immunoprecipitated using an anti-Myc antibody, and immunoprecipitates were blotted with an anti-HA antibody. (B) HEK-293T cells were transfected with HA-Ubiquitin, Myc-NEMO, OMNI-RIP2, GFP-CYLD WT or S418A, and GST-IKKε. NEMO was immunoprecipitated with an anti-Myc antibody and immunoprecipitates were blotted with an anti-HA antibody.

phosphorylation of CYLD leads to decreased deubiquitinase activity. As in Figure 3.10A cotransfection of CYLD S418A with IKKε did not lead to suppression of NEMO ubiquitination (Figure 3.10B). Together, these observations demonstrate that phosphorylation of CYLD at Ser418 leads to decreased CYLD deubiquitinase activity.

We next sought to determine what effect this phosphorylation exerts on NF-κB activity. We have shown previously that MCF-7 cells express elevated levels of endogenous IKKε [17]. These cells were transiently transfected with Myc-TRAF2 alone or in combination with Myc-CYLD, Myc-CYLD S418A, or Myc-CYLD S772A, and we performed a NF-κB-luciferase reporter assay. In cells expressing TRAF2 alone, we found substantial (14.9 fold) activation of the NF-κB reporter (Figure 3.11A). Co-transfection with a sub-saturating amount of WT CYLD or CYLD S772A resulted in an approximately 50% decrease in TRAF2-induced NF-κB activation. However, co-transfection with CYLD S418A resulted in a statistically significantly larger decrease in NF-κB reporter activity ($p<0.05$) (Figure 3.11A). These results demonstrate that phosphorylation of CYLD at Ser418 suppresses CYLD activity, which in turn results in an increase in NF-κB transcriptional activation.

To further validate this observation, we assessed whether CYLD phosphorylation by IKKε modulates NF-κB activity in the context of transformation. NF-κB luciferase reporter activity was measured in IKKε-transformed NIH-3T3 cells overexpressing WT or mutant CYLD (S418A and S772A). IKKε-transformed cells exhibited strong NF-κB activation in comparison to control cells, and the introduction of WT CYLD and S772A failed to induce a substantial change in NF-κB activity

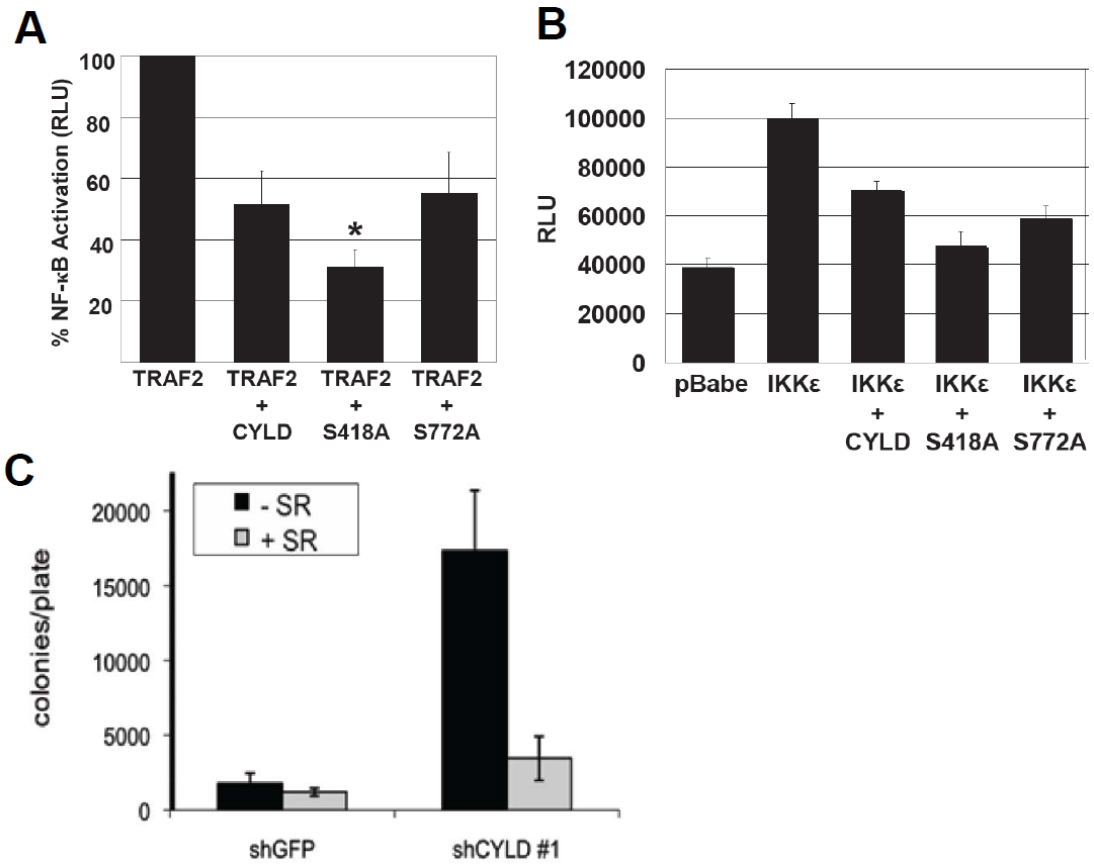

Figure 3.11. Phosphorylation of CYLD at Ser418 decreases CYLD activity. (A) MCF-7 cells were transfected with an NF-κB–luciferase reporter gene, CMV-driven *Renilla* luciferase (to standardize transfection efficiency), and TRAF2 (200 ng) either alone or in combination with a subsaturating amount of Myc-CYLD WT, Myc-CYLD S418A, or Myc-CYLD S772A (5 ng). An NF-κB–luciferase reporter assay was performed at 24 h posttransfection. Each experiment was performed four times with similar results. TRAF2 alone increased NF-κB reporter activation 14.9 fold. CYLD S418A inhibited the NF-κB response induced by TRAF2 more efficiently than WT CYLD or CYLD S772A. Error bars represent SD. Astericks indicates statistical significance ($p<0.05$). (B) IKKε-transformed NIH-3T3 cells stably expressing WT or mutant CYLD were transiently transfected with pTRH1-NF-κB-Luciferase reporter construct in parallel to pRL-SV40-Renilla. Additional ectopic WT and mutant CYLD was also introduced in these experiments as described in the Materials and Methods. Luciferase activity was assessed 2 days post transfection. Representative of 4 separate experiments. Error bars represent SD. (C) Anchorage-independent growth of NIH-3T3 cells was assessed following suppression of endogenous CYLD with shCYLD1 and simultaneous expression of an NF-κB "superrepressor" (IκBα S32,36A) for 21 days. Error bars represent SD for 3 independent experiments.

(Figure 3.11B). As observed in MCF-7 cells, however, CYLD S418A more strongly inhibited IKKε-induced NF-κB activation. These findings indicate that IKKε-mediated phosphorylation of CYLD at serine 418 plays a role in the observed activation of NF-κB. As the decrease in NF-κB activation observed in the presence of CYLD S418A was not as robust as the decrease in transformation, it is likely that NF-κB is not the only pathway affected by phosphorylation of CYLD. However, these findings suggest that increased the regulation of CYLD phosphorylation by IKKε contributes to the NF-κB activity necessary for IKKε-mediated cell transformation.

To further confirm that cell transformation mediated by CYLD suppression was NF-κB dependent, we utilized the NIH-3T3 cells expressing shRNAs targeting murine CYLD which were described in Figure 3.8. When a non-phosphorylatable IκBα mutant (IκBα S32,36A), which acts as an NF-κB "superrepressor," was stably expressed in these cells, we found a dramtatic reduction in anchorage-independent colony growth (Figure 3.11C). Thus, suppression of CYLD induces NF-κB-dependent cell transformation similar to that induced by overexpression of IKKε.

DISCUSSION

IKKε plays a central role in regulating innate immunity and also acts as an oncogene in a significant fraction of breast cancers. To gain a deeper understanding of the role(s) of IKKε in these biological processes, we used an unbiased proteomic and bioinformatic approach, which allowed us to identify the optimal substrate phosphorylation motif for IKKε. Based on these findings, a proteome-wide search for

sites that correspond to the IKKε phosphorylation motif then led to the identification of a large number of putative IKKε substrates.

Of the candidate IKKε substrates identified, many have known roles in inflammatory and/or oncogenic signaling pathways (Table 3.2). In particular, serine 418 of the deubiquitinase and tumor suppressor CYLD emerged as a very likely site of IKKε phosphorylation. Here we showed that IKKε directly phosphorylates CYLD and have used mass spectrometry and an IKKε phospho-substrate antibody to show that Ser418 of CYLD is, in fact, the *in vivo* site phosphorylated by IKKε. While it has been reported previously that IKKα and IKKβ are capable of phosphorylating Ser418 of CYLD [37], we demonstrated that CYLD is phosphorylated much more efficiently by IKKε than by either of the canonical IKKs.

Upon finding that an oncoprotein is capable of strongly phosphorylating a known tumor suppressor, we predicted that CYLD phosphorylation at Ser418 might play an important role in cell transformation. Indeed, we found that introduction of CYLD S418A suppressed anchorage-independent growth in IKKε-transformed NIH-3T3 cells and hindered IKKε-driven tumor growth. The data presented here therefore define the one potential mechanism by which IKKε induces tumorigenesis. Further examination showed that phosphorylation of CYLD at S418 decreases its deubiquitinase activity and increases NF-κB activation. Activation of NF-κB therefore appears to be one important mechanism by which CYLD phosphorylation regulates cell transformation. As CYLD also has functions independent of NF-κB it is likely that other signaling pathways also may be involved. Moreover, we recognize that IKKε may also modulate other substrates in addition to CYLD during cell

transformation. Thus, although further work will be necessary to evaluate the full mechanisms by which IKKε and CYLD contribute to transformation, these observations connect an oncogene and tumor suppressor that both regulate NF-κB to cell transformation.

The role of chronic inflammation in the development of inflammation-related cancers is well-known, but the mechanisms by which inflammatory mediators cause cell transformation are poorly understood [1-3]. For kinases involved in these signaling pathways, such as the anti-viral mediator and oncoprotein IKKε, our inability to identify the complete repertoire of intracellular substrates has hindered progress towards understanding how these enzymes exert their oncogenic effects. The techniques described here, involving the identification of the phosphorylation motif for a kinase, followed by development of a phospho-substrate antibody and bioinformatic prediction of substrates, can provide an efficient and unbiased method for the identification and validation of physiologically relevant kinase substrates. We have validated this method through the identification of CYLD as a novel IKKε substrate and have shown that CYLD phosphorylation is necessary for full IKKε-mediated anchorage-independent growth. As expression of CYLD S418A does not completely suppress IKKε-driven cell transformation, CYLD is unlikely to be the only IKKε substrate important for cell transformation. However, the strength of the approach described here, as opposed to more targeted methods of substrate identification, is that it has simultaneously identified a large number of other potentially important IKKε substrates. Available *in vitro* assays should allow other putative substrates to be efficiently tested for their ability to modulate IKKε-driven

cell transformation, leading to both an increased understanding of how IKKε controls oncogenesis, and, perhaps, new insights into therapeutic targets for IKKε-driven cancers.

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CHAPTER FOUR

IKKε PHOSPHORYLATES TRAF2 TO PROMOTE MAMMARY EPITHELIAL CELL TRANSFORMATION

IKKε phosphorylates TRAF2 to promote mammary epithelial cell transformation

This chapter is adapted from the following manuscript:

Rhine R. Shen, Alicia Y. Zhou, Eejung Kim, William C. Hahn. IKKε phosphorylates TRAF2 to promote mammary epithelial cell transformation. *Submitted*.

Contributions: Rhine R. Shen and William C. Hahn wrote the manuscript. R. Shen, Alicia Y. Zhou and William C. Hahn conceived and designed the experiments. Rhine R. Shen and Alicia Y. Zhou performed the experiments with technical assistance from Eejung Kim. Specific contributions: Alicia Y. Zhou produced the data shown in Figures 4.5A, 4.6 and 4.7, analyzed the data shown in Figures 4.4B and 4.10, and adapted the manuscript for this chapter.

ABSTRACT

NF-κB transcription factors are central regulators of inflammation and when dysregulated contribute to malignant transformation. IκB kinase ε (IKKε, IKKi, *IKBKE*) is a breast oncogene that is amplified and overexpressed in 30% of breast cancers and drives transformation in a NF-κB-dependent manner. Here we identify TRAF2 as an oncogenic substrate of IKKε. IKKε interacts with and phosphorylates TRAF2 at Ser11 *in vitro* and *in vivo*. This activity promotes Lys63-linked TRAF2 ubiquitination, NF-κB activation and is essential for IKKε-transformation. Breast cancer cells that depend on IKKε expression for survival are also dependent on TRAF2. This work defines TRAF2 phosphorylation as a key component of IKKε-induced mammary epithelial cell transformation.

INTRODUCTION

NF-κB activation plays a key role in innate immunity and inflammation. Several lines of evidence indicate that dysregulation of NF-κB signaling also contributes to malignant transformation through both cell autonomous and cell non-autonomous mechanisms. Although cancers associated with chronic inflammation are frequently dependent on aberrant NF-κB activity induced by the tumor microenvironment [1-4], somatic mutations in components of the NF-κB pathway that lead to constitutive NF-κB activation also contribute directly to tumorigenicity.

Activation of the canonical NF-κB pathway is facilitated by the recruitment of receptor associated adaptor molecules such as TNF receptor associated factors, TRAFs [5]. TRAF proteins mediate the formation of protein complexes that activate the classical IκB kinase (IKK) complex, consisting of the catalytic kinases IKKε and IKKε, and the regulatory subunit IKKγ/NEMO. IKK complex activation triggers proteasomal-mediated degradation of the key inhibitory molecule IκBα that, in turn, permits nuclear translocation of NF-κB dimers. In addition to this canonical mode of activation in response to TNF, an array of inflammatory stimuli activate several signaling pathways that converge to activate NF-κB [6].

IKKε (IκB kinase ε, encoded by *IKBKE*) is a non-canonical IKK family member that activates both interferon and NF-κB signaling. In response to viruses, both IKKε and another non-canonical IKK, TBK1, form a complex to phosphorylate interferon regulatory factors 3 and 7 (IRF3 and IRF7) [7]. This activity is essential for the nuclear translocation of IRF3 and IRF7 and transcriptional activation of type I

interferon genes [8]. In addition to its role in innate immunity, *IKBKE* is also a breast oncogene that is amplified and overexpressed in approximately 30% of breast cancers [9]. Suppression of *IKBKE* induces apoptosis in breast cancer cell lines that harbor increased *IKBKE* copy number. IKKε overexpression induces malignant transformation in immortalized human and murine cells in a NF-κB dependent manner [9].

We recently employed a scanning peptide library screen and Scansite bioinformatic analysis to identify an IKKε kinase recognition motif and potential IKKε substrates. Using this combined proteomic and bioinformatic approach, we identified the familial tumor suppressor CYLD to be an IKKε substrate involved in cell transformation [Chapter 3, 10]. As a deubiquitinase, CYLD acts as a negative regulator of NF-κB signaling. Phosphorylation of CYLD by IKKε at serine 418 inactivates CYLD function and leads to NF-κB activation. Although this phosphorylation event was necessary for IKKε-mediated transformation, cells rendered tumorigenic by IKKε expression were only partially dependent on CYLD for transformation. These observations suggested that IKKε regulates other effectors that participate in NF-κB pathway activation and transformation.

Here we identify TRAF2, an adaptor molecule that assembles active NF-κB signaling modules, as a substrate of IKKε. IKKε phosphorylates TRAF2 at Ser11 and this activity is required for IKKε-induced NF-κB activation and transformation.

MATERIALS AND METHODS

Antibodies, Plasmids, and Reagents. The antibodies used include: Myc (clone 4A6) (Millipore), HSP90, Lamin A/C, p50, p52, CIAP1, TRAF2 (Rabbit) and TANK (Cell Signaling Technologies), V5-HRP (Invitrogen), Ubiquitin (Santa Cruz Biotechnology), IKKε and β-actin (Sigma-Aldrich), HA (Clone12C5) (Boehringer Mannheim), a mouse monoclonal TRAF2 antibody (Imgenex). The IKKε phospho-substrate antibody was previously described [10]. The phospho-TRAF2 (Ser 11) antibody was a gift from Dr. Hasem Habelhah. Anti-V5 affinity gel agarose was obtained from Sigma- Aldrich.

Myc-TRAF2 was created by PCR cloning into the BamHI site of the 3XMyc (pEBB) vector. GST-IKKε, GST-K38A, and have been described [10]. Flag-IKKε and Myr-Flag-IKKε were used as described [9]. Myc-TRAF2 S11A, Myc-TRAF2 S102A, Myc-TRAF2 S274A, Myc-TRAF2 S327A, and Myc-TRAF2 S408A and were created using QuickChange site-directed mutagenesis protocol (Stratagene). pWZL-TRAF2 WT and site-directed mutants were created by subcloning into the BamHI site of the pWZL-Blast retroviral expression vector(s). V5-GFP, V5-TRAF2, V5-TRAF2 S11A, and V5-TRAF2 S102A were generated by gateway cloning into the pLEX-V5-Blast lentiviral construct. HA-ubiquitin, HA-Ub K63-only, and HA-Ub K48-only were used as described previously [9, 11]. shIKKε and shCYLD lentiviral constructs have been described [9, 10]. Additional shRNA constructs were obtained from the RNAi Consortium (Broad Institute) and include pLKO-shTRAF2#1, pLKO-shTRAF#2, pLKO-shGFP, pLKO-shRFP, pLKO-shLacZ, and pLKO-shLuc.

Cell Culture, Transfection, Immunoprecipitation, Subcellular Fractionation, and Immunoblotting. HEK293T, MDA-MB-453, MCF-7, RKO cells were obtained from ATCC and were grown in DMEM containing 10% FBS. NIH3T3 cells were obtained from ATCC and grown in DMEM containing 10% bovine calf serum. HA1EM and HMLEM cells have been described [9]. BT549, BT474, MCF-7, MDA-MB-453, T47D, and ZR-75-1 were obtained from ATCC and maintained in RPMI1640 containing 10% FBS. SUM52 cells were maintained in HamsF-12:MEGM(1:1) with 10% FBS. Transfection experiments were performed using Fugene (Roche). Immunoprecipitations and immunoblotting experiments were conducted as described [12, 13].

***In vitro* kinase assays.** Recombinant GST-IKKε (Invitrogen) and recombinant GST-TRAF2 (Novus Biologicals) were used. Kinase buffer contained 50 mM Tris (pH 7.5), 12 mM $MgCl_2$, 1 mM β-glycerophosphate, 100 μM ATP, and 10 μCi γ-^{32}P-ATP/reaction. Reactions were incubated at 30ºC for 1h, resolved on SDS-PAGE, and directly exposed to radiography film.

NF-κB reporter assays

HA1EM and HEK293T cells were transfected with pTRH1-NF-κB-Luciferase reporter NF-κB-luciferase reporter (System Biosciences) in parallel to pRL-SV40-Renilla (Promega). NF-κB activity was measured using the Dual-Glo Luciferase assay (Promega) 36h post-transfection. Alternatively, GloResponse NF-κB-RE-*luc2P* HEK293T cells (Promega) were transfected as indicated and NF-κB activity was

measured 36h post-transfection according to the protocol for the One-Glo Luciferase assay (Promega). Luciferase values were normalized to renilla values to yield relative light units (RLU).

In vitro cell transformation assay

Growth of NIH 3T3, HA1EM, or HMLE cells in soft agar was determined by plating 5 X 10^4 cells in triplicate in 0.4% Noble agar. Microscopic (greater than 100μm in diameter) or macroscopic (greater than 1500μm in diameter) colonies were counted 28 d after plating.

Viability and proliferation measurements

Proliferation assays were performed in duplicate using a Vi-Cell counter every 7d for 14 d and plotted as a function of population doubling (PD) vs. time. PD were defined as [$\log_2$(cells counted/cells plated)].

RESULTS

IKKε phosphorylates and binds to TRAF2

We previously demonstrated IKKε phosphorylates and inactivates the tumor suppressor CYLD using an integrated proteomic and bioinformatic approach [Chapter 3, 10]. Although CYLD is one IKKε substrate that contributes to cell transformation, we found that suppression of CYLD failed to fully inhibit IKKε-mediated tumorigenicity, suggesting that other IKKε effectors contribute to cell transformation.

When we reexamined the list of candidate IKKε substrates, we recognized that TRAF2, a molecule that plays an essential role in TNF-induced NF-κB activation, harbors several potential IKKε substrate recognition motifs. To test whether IKKε phosphorylates TRAF2, we performed an *in vitro* kinase assay using recombinant GST-tagged IKKε and recombinant GST-tagged TRAF2. We observed robust autophosphorylation of IKKε and also verified that TRAF2 was strongly phosphorylated by IKKε (Figure 4.1A). To confirm that IKKε phosphorylates TRAF2 *in vivo*, we first co-expressed a Myc-epitope tagged TRAF2 and either GST-tagged IKKε or kinase-inactive IKKε K38A in human embryonic kidney epithelial cells (HEK293T). We then isolated TRAF2 immune complexes and found that TRAF2 was phosphorylated by wildtype IKKε in a kinase-dependent manner using an IKKε phospho- substrate antibody (Figure 4.1B). These observations demonstrate that IKKε phosphorylates TRAF2 both *in vitro* and *in vivo*.

We next examined whether IKKε interacts with TRAF2. In the HEK293T cells co-expressing Myc-TRAF2 and GST-IKKε or the kinase inactive mutant GST-IKKε K38A, we found that both WT and K38A IKKε and TRAF2 formed a readily detectable complex (Figure 4.1B). To confirm that this interaction occurs under conditions where IKKε expression induces cell transformation, we determined whether IKKε interacts with endogenous TRAF2 in immortal but non-tumorigenic human embryonic kidney cells (HEK) expressing hTERT, SV40 Large T and small t oncoproteins, and a constitutively active MEK^{DD} allele (HA1EM) [9]. Using these

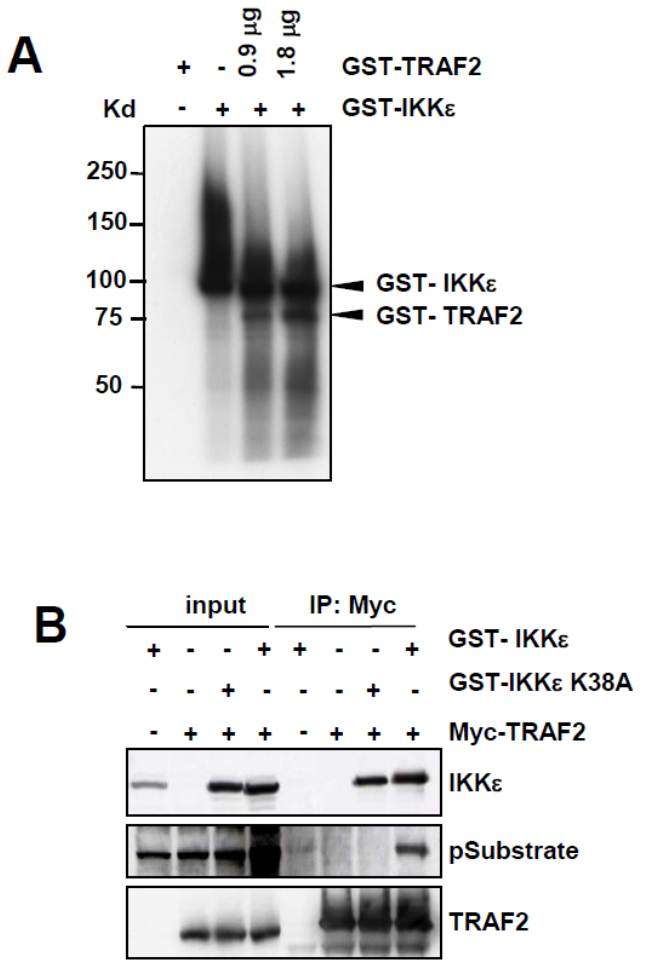

Figure 4.1. IKKε phosphorylates TRAF2 *in vitro* and *in vivo*
(A) *In vitro* phosphorylation of TRAF2 by IKKε. Kinase assay using recombinant GST-IKKε and GST-TRAF2. Two concentrations (0.9 µg and 1.8 µg) of GST-TRAF2 were phosphorylated by GST-IKKε. (B) Phosphorylation of TRAF2 by IKKε in HEK293T cells. Myc–TRAF2 immune complexes were isolated from HEK293T cells expressing the indicated proteins and analyzed by immunoblot with antibodies specific for IKKε, IKKε phospho-substrates, and TRAF2.

cells, we isolated endogenous TRAF2 immune complexes and verified that IKKε and TRAF2 interact (Figure 4.2A).

TRAF2 has been shown to form a complex with the ubiquitin ligase CIAP1, the TRAF family member-associated NF-κB activator (TANK) and TBK1, a non-canonical IKK closely related to IKKε [14, 15]. This complex acts as a scaffold that facilitates the activation of NF-κB signaling. To determine whether CIAP1 and TANK were also present in the complex formed by IKKε and TRAF2, we isolated TRAF2 immune complexes from HA1EM cells expressing V5-tagged TRAF2 and detected endogenous IKKε, TBK1, CIAP1 and TANK (Figure 4.2B). We also assessed whether this complex forms in IKKε-transformed HA1EM cells. Specifically, we isolated TANK immune complexes from HA1EM cells and HA1EM cells stably expressing Flag-epitope tagged IKKε (HA1EM-F-IKKε) and found a complex composed of endogenous TANK, TRAF2, CIAP1, TBK1 and IKKε (Figure 4.2C).

Overexpression of IKKε increased the abundance of the complex, suggesting that IKKε drives the recruitment of these components. To further verify these observations, we examined whether this complex exists in breast cancer cells that depend on IKKε for survival. Specifically, we confirmed that the IKKε-TANK-TRAF2-CIAP1-TBK1 is present in MCF-7 cells (Figure 4.2D). These findings provide evidence that IKKε forms a complex with TRAF2, TANK, TBK1 and CIAP1.

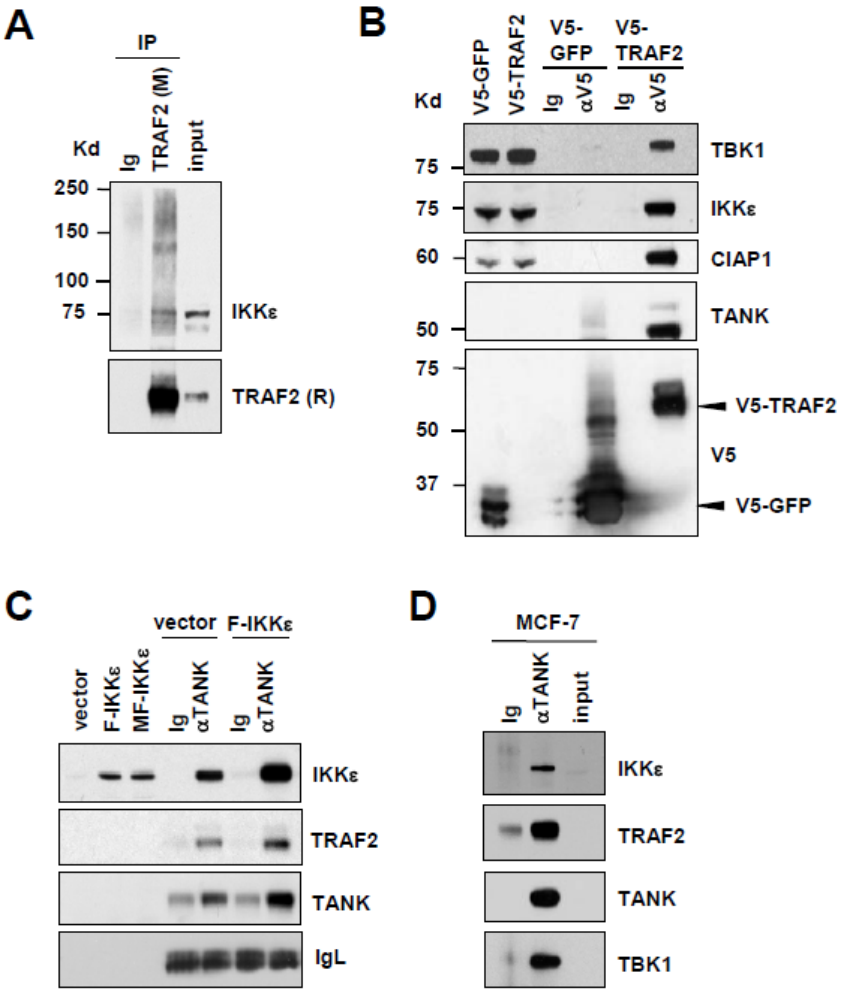

Figure 4.2. IKKε forms a complex with TRAF2, CIAP1, TANK and TBK1
(A) Endogenous interaction between IKKε and TRAF2 in IKKε -transformed cells. TRAF2 immune complexes were isolated from HA1EM cells using a murine (M) monoclonal antibody and analyzed by immunoblot with IKKε and rabbit (R) TRAF2 antibodies. (B) TRAF2, IKKε, TANK, CIAP1 and TBK1 form a complex. V5 immune complexes were isolated from HA1EM cells transduced with V5-GFP or V5-TRAF2 and analyzed by immunoblot with antibodies specific for V5, TANK, CIAP1, IKKε, or TBK1. Murine immunoglobulin (mIg) was used for control immunoprecipitations. (C) Complex formation of endogenous TANK, TRAF2, and IKKε. TANK immune complexes were isolated from HA1EM cells transduced with control vector or F-IKKε and analyzed by immunoblot with antibodies specific for IKKε, TRAF2, and TANK. Rabbit Ig (rIg) was used for control immunoprecipitations. (D) Complex formation of endogenous TANK, TRAF2, IKKε and TBK1 in MCF-7 cells. TANK immune complexes were isolated from MCF-7 breast cancer cells and analyzed by immunoblot with antibodies specific for IKKε, TRAF2, TBK1 and TANK. Rabbit Ig (rIg) was used for control immunoprecipitations.

IKKε phosphorylates TRAF2 on Ser11 to facilitate NF-κB activation

To identify TRAF2 residue(s) that are phosphorylated by IKKε, we surveyed the TRAF2 protein sequence for serine residues that correspond to the IKKε kinase recognition motif [10]. This motif consists of a central serine phosphorylation site neighbored by a leucine residue at the +1 position and hydrophobic residues at the -2 and +3 positions (Figure 4.3A). We found five TRAF2 serine residues at positions 11, 102, 274, 327 and 408 that matched this motif.

We then interrogated whether these sites are phosphorylated by IKKε by generating serine to alanine substitutions for each putative target serine residue. After coexpressing GST-IKKε with wildtype or each of these TRAF2 mutants in HEK293T cells, we isolated TRAF2 immune complexes and found that each of the TRAF2 mutants retained the ability to bind IKKε (Figure 4.3B). However, when we examined the phosphorylation status of wildtype and mutant TRAF2, we found that IKKε phosphorylated TRAF2 S102A, TRAF2 S274A, TRAF2 S327A and TRAF2 S408A comparably to wildtype TRAF2. In contrast, we found that IKKε failed to phosphorylate TRAF2 S11A (Figure 4.3B), indicating that IKKε targets TRAF2 Ser11 for phosphorylation.

To examine whether IKKε phosphorylates endogenous TRAF2, we investigated the consequences of manipulating IKKε expression on the phosphorylation of TRAF2 at Ser11. First, we suppressed IKKε in HA1EM cells and several breast cancer cell lines that depend on IKKε for tumorigenic growth (MCF-7, ZR-75-1 and MDA-MB-453). We introduced two distinct IKKε-specific shRNAs in these cells and measured TRAF2 phosphorylation at Ser11 using a phospho-TRAF2

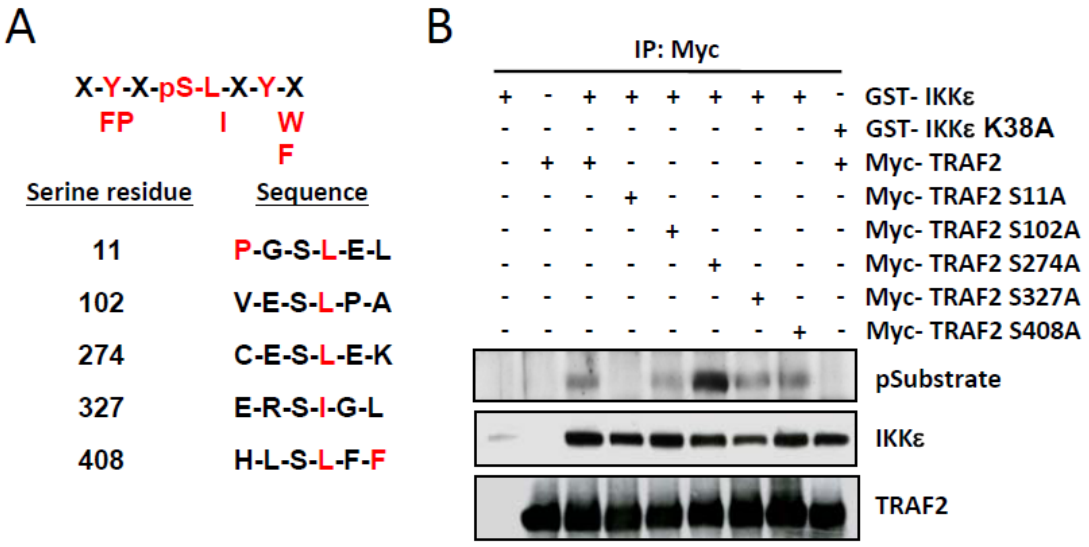

Figure 4.3. IKKε phosphorylates TRAF Ser11.
(A) IKKε recognition motif and candidate TRAF2 serine residues that match the motif. Highlighted residues (red) indicate a match to the recognition motif. (B) IKKε binds to and phosphorylates TRAF2 at Ser 11. HEK293T cells were transfected as indicated. Myc-TRAF2 immune complexes were isolated and analyzed with antibodies specific for TRAF2, IKKε, and IKKε phospho-substrates.

(Ser11)-specific antibody [16]. Suppression of IKKε in HA1EM cells with these two IKKε-specific shRNAs resulted in a 3.6- and 4.2-fold decrease in the levels of TRAF2 Ser11 phosphorylation, respectively (Figure 4.4A). We also observed a decrease in TRAF2 Ser11 phosphorylation after IKKε suppression in MCF-7, ZR-75-1 and MDA-MB-453 breast cancer cells (Figure 4.4B). To corroborate these observations, we determined whether expression of IKKε promoted an increase in TRAF2 phosphorylation at Ser11. We previously showed that the introduction of F-IKKε or Myristoylated, Flag-epitope tagged IKKε (MF-IKKε) transforms HA1EM cells [9]. When we assessed the levels of phosphorylated TRAF2 in HA1EM expressing F-IKKε or MF-IKKε, we found a 2.3 and 4.8-fold increase respectively in TRAF2 Ser11 phosphorylation (Figure 4.4C). These observations confirmed that IKKε phosphorylates TRAF2 at Ser11 *in vivo* and suggest that this activity is important for cell transformation.

To elucidate the functional consequences of IKKε-mediated TRAF2 Ser11 phosphorylation, we assessed whether this activity modulates NF-κB activation. Using GloResponse NF-κB-RE-*luc2P* HEK293T cells (Promega), a cell line that stably expresses a NF-κB luciferase reporter, we found that expression of either IKKε or TRAF2 induced a 5- fold or 14-fold increase in NF-κB activity respectively (Figure 4.5A, [9, 16]). In contrast, both kinase inactive IKKε K38A and TRAF2 S11A failed to promote NF-κB activation (Figure 4.5A). To evaluate the effects of IKKε and TRAF2 induced NF-κB activation in cells that are readily transformed by

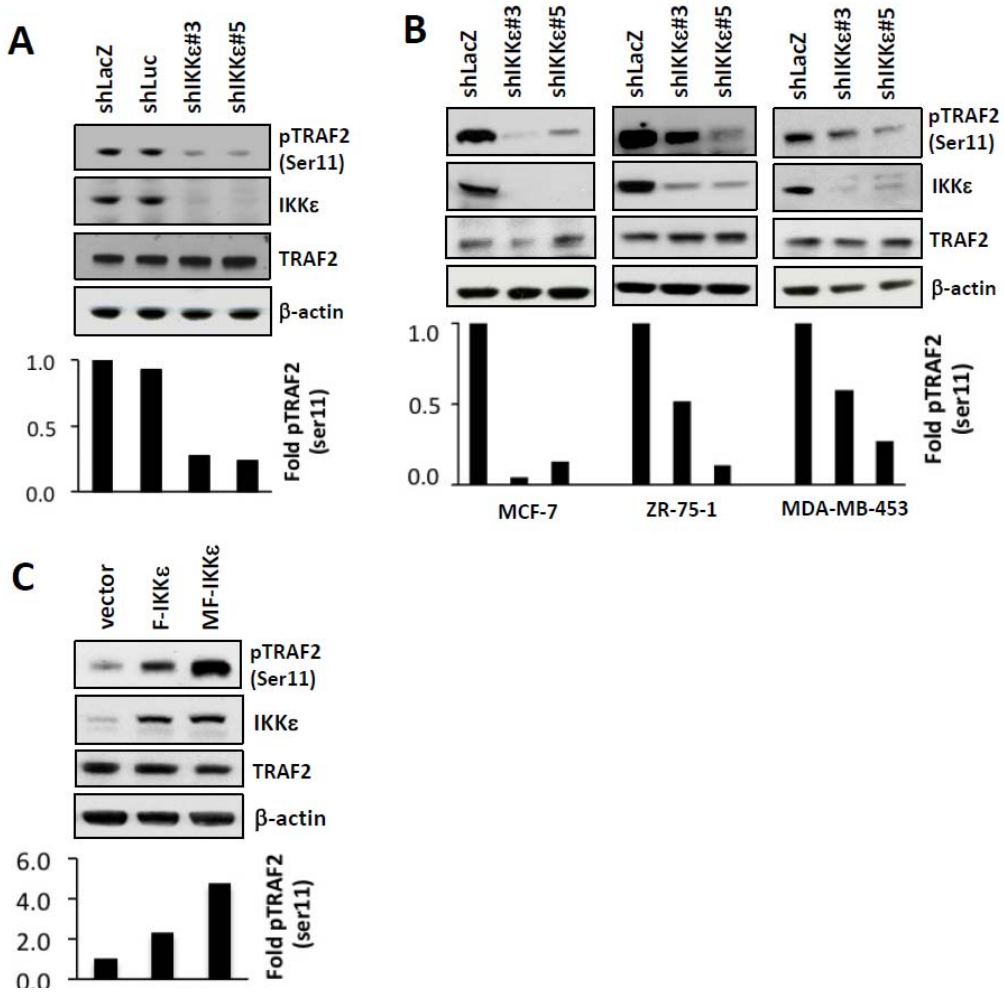

Figure 4.4. IKKε phosphorylates TRAF2 *in vivo*
(A and B) Effects of suppressing IKKε on TRAF2 Ser11 phosphorylation. Immunoblot of phospho-TRAF2 (Ser11) in HA1EM cells(A) or in MCF-7, ZR-75-1, and MDA-MB-453 breast cancer cells(B) transduced with shLacZ or shLuc controls or two *IKKε*-specific shRNAs (shIKKε #3 and shIKKε #5). Total IKKε, TRAF2 and β-actin expression are shown. Densitometry analysis for is shown in the lower panel. (C) TRAF2 Ser11 phosphorylation in IKKε -transformed HA1EM cells. Immunoblot was performed as in (A) on HA1EM cells expressing a control vector, F-IKKε, MF-IKKε. Densitometry analysis is shown in the lower panel.

IKKε, we performed NF-κB luciferase reporter experiments in HA1EM cells. Expression of either IKKε or TRAF2 alone in HA1EM cells induced a 2.7-fold increase in NF-κB activity as compared to the activity measured in cells expressing a control vector (Figure 4.5B). In contrast, coexpression of IKKε and wildtype TRAF2 resulted in a 7-fold increase in NF-κB activity. Since we failed to observe a similar increase in IKKε-mediated NF-κB activation by coexpressing IKKε and TRAF2 S11A, we concluded that the increased NF-κB activity was due to IKKε -mediated phosphorylation of TRAF2 at Ser11. In consonance with these findings, we found that wildtype TRAF2 induced the expression of NF-κB target genes BIRC3 and IL-6 by 2.5 and 4.3 fold respectively in MCF-7 cells, whereas TRAF2 S11A did not significantly activate these NF-κB regulated genes (Figure 4.5C). Collectively, these results suggest that IKKε-mediated TRAF2 Ser11 phosphorylation facilitates downstream NF-κB activation.

IKKε-induced TRAF2 Ser11 phosphorylation promotes TRAF2 Lys63-linked ubiquitination

Ubiquitination plays a key role in NF-κB activation [17, 18]. For example, Lys48-linked ubiquitination mediates proteasomal degradation of IκBα that is essential for nuclear translocation of NF-κB transcription factors [19]. In addition, modification of NF-κB regulators including TRAFs, RIP and NEMO with Lys63-linked ubiquitin chains facilitates the formation of a protein platform consisting of the canonical IKKs

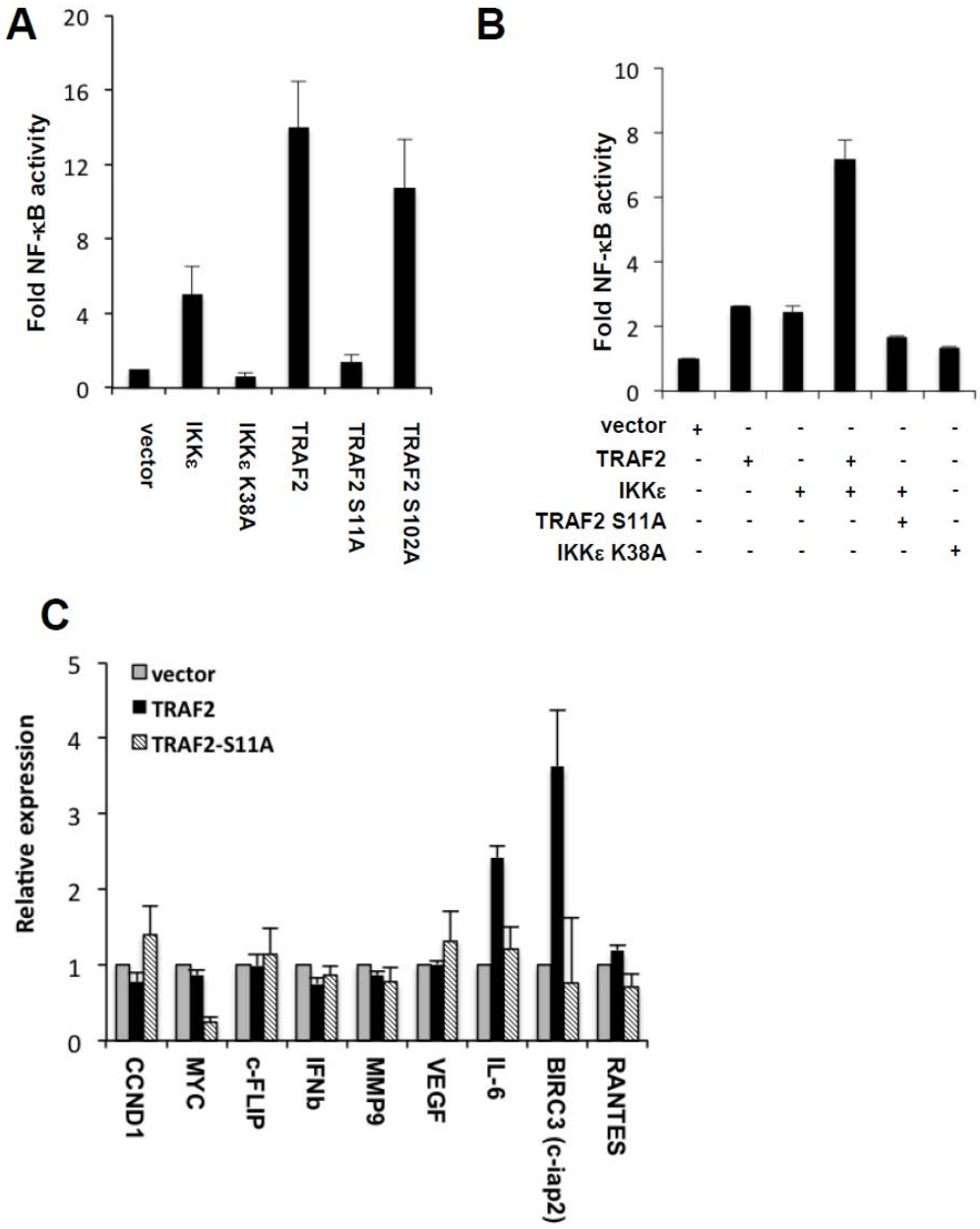

Figure 4.5. IKKε promotes TRAF2 induced NF-κB activation
(A) IKKε and TRAF2 induced NF-κB activation. GloResponse NF-κB-RE-*luc2P* HEK293T luciferase reporter cells were transfected as indicated and raw light unit (RLU) activity was normalized to activity observed with control vector. (B) IKKε-induced NF-κB activation in the presence of wildtype TRAF2 and TRAF2 S11A. HA1EM cells were cotransfected as indicated with SV40-renilla, and a NF-κB luciferase. Raw light unit (RLU) activity was normalized to activity observed with control vector. Results reported as mean ± SD of three experiments. (C) Change in expression of selected NF-κB regulated genes in MCF-7 cells harboring either a control vector, TRAF2 or TRAF2 S11A

as well as the IKK kinases, TAK1, RIP1 and other scaffolding molecules [17, 18]. Prior work has shown that TRAF2 is modified by both Lys63- and Lys48-linked ubiquitination, and that these modifications regulate the recruitment of the IKK complex and downstream NF-κB activation [20]. We reasoned that TRAF2 phosphorylation by IKKε may regulate TRAF2 ubiquitination and evaluated total ubiquitination of TRAF2 in the presence and absence of IKKε. We coexpressed Myc-TRAF2, HA-Ubiquitin, and either GST-IKKε or GST-IKKε K38A in HEK293T cells. From these cells, we isolated TRAF2 immune complexes and determined whether TRAF2 was ubiquitinated. We found that expression of IKKε dramatically increased TRAF2 ubiquitination in a kinase-dependent manner, suggesting that phosphorylation of TRAF2 by IKKε was required for TRAF2 ubiquitination (Figure 4.6A). We made similar observations in both IKKε-transformed human mammary epithelial cells (HMLEM) and HA1EM cells stably expressing Flag-TRAF2. In both cases, when we isolated TRAF2 immune complexes, we found ubiquitin-containing species in cells overexpressing IKKε (Figure 4.6B and C). These observations indicate that IKKε promotes TRAF2 ubiquitination in transformed cells.

To confirm that IKKε regulates TRAF2 ubiquitination specifically through phosphorylation of Ser11, we evaluated the ubiquitination status of both wildtype TRAF2 and TRAF2 S11A. When we expressed wildtype Myc-TRAF2 in HEK293T cells and performed a TRAF2 immunoblot, we observed a series of high molecular weight species that correspond to TRAF2 ubiquitination. In contrast, Myc-TRAF2 S11A expression results in a substantial decrease in TRAF2 ubiquitination. Interestingly, a catalytically inactive form of TRAF2, in which the E3 ligase RING

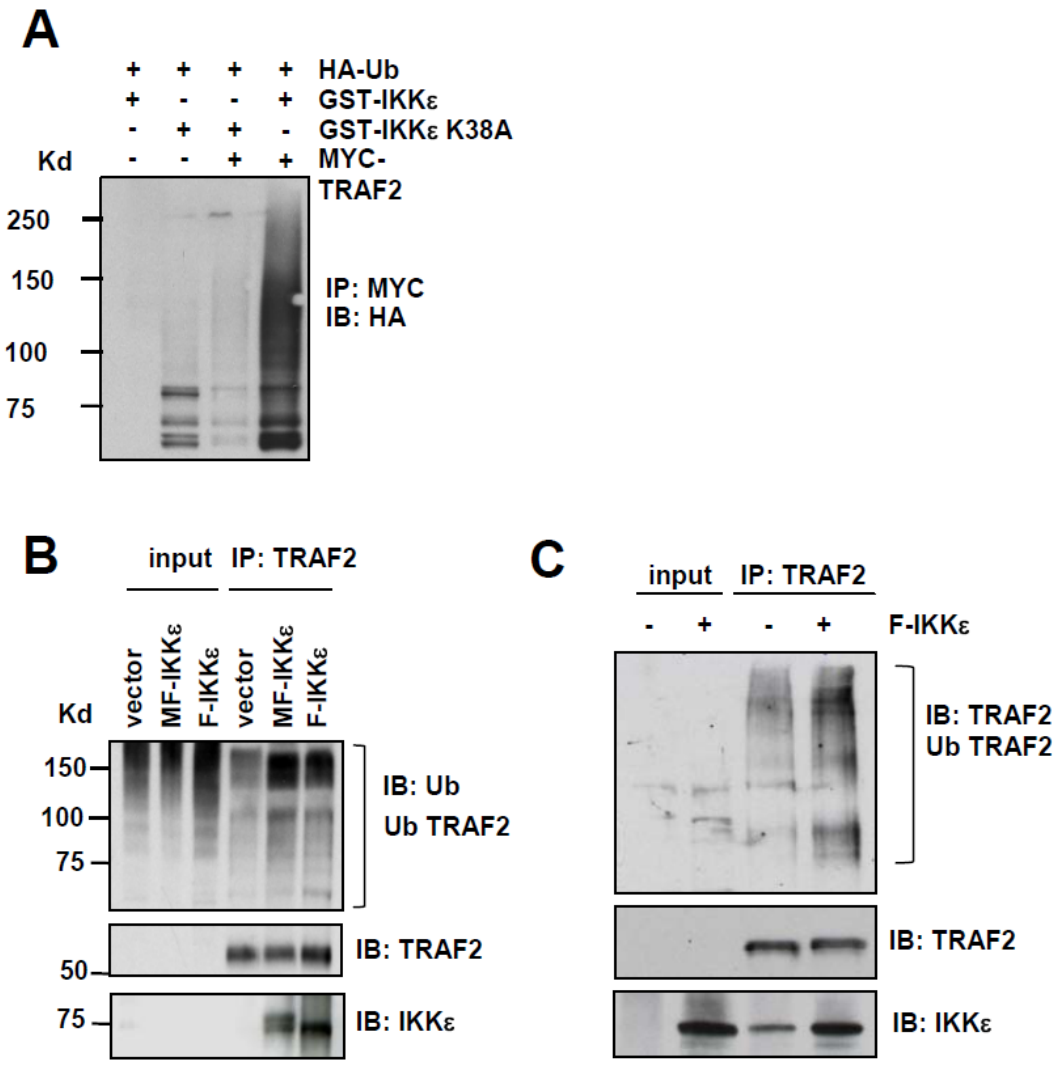

Figure4.6 IKKε promotes TRAF2 ubiquitination

(A) Total TRAF2 ubiquitination in HEK293T cells cotransfected as indicated with HA-Ub. Myc-TRAF2 immune complexes were isolated and analyzed by immunoblot with an HA antibody. (B) Endogenous TRAF2 ubiquitination in HMLEM cells expressing a control vector, MF-IKKε and F-IKKε. TRAF2 immune complexes were isolated and analyzed by immunoblot with antibodies specific for ubiquitin, TRAF2 and IKKε. (C) Endogenous TRAF2 ubiquitination in HA1EM cells expressing a control vector and F-IKKε. TRAF2 immune complexes were isolated and analyzed by immunoblot with antibodies specific for TRAF2 and IKKε.

domain of the protein has been deleted (ΔRING), did not undergo ubiquitination at all., suggesting that the sites of TRAF2 ubiquitination are found within the RING domain of the protein (Figure 4.7A). In agreement with our prior findings, we found that coexpression of V5-IKKε and Myc-TRAF2 expression induces in a dramatic increase in TRAF2 Ser11 phosphorylation. Moreover, the phosphorylated TRAF2 is strongly ubiquitinated in the presence of IKKε (Figure 4.7B). In contrast, kinase inactive IKKε K38A failed to promote either phosphorylation or ubiquitination of TRAF2. These findings demonstrate that Ser11 phosphorylation by IKKε is important for TRAF2 ubiquitination.

We next determined which type of ubiquitin linkages were affected by TRAF2 phosphorylation. Specifically, we co-expressed a HA-tagged ubiquitin mutant that only contains Lys63 linkages (HA-Lys63-Ub) and either wildtype IKKε or IKKε K38A in HEK293T cells, isolated HA-Ubiquitin immune complexes, and determined whether TRAF2 was present. We found that the expression of wildtype but not kinase inactive IKKε induced a 3-fold increase in Lys63-linked ubiquitination of TRAF2 (Figure 4.7C). These observations demonstrate that IKKε-induced ubiquitination of TRAF2 involves Lys63-linked ubiquitin and is dependent on IKKε kinase activity.

To determine whether TRAF2 Lys63-linked ubiquitination requires Ser11 phosphorylation, we introduced the Myc-TRAF2 mutants (S11A and S102A) and HA-Lys63-Ub in HEK293T cells. We then isolated HA-ubiquitin immune complexes and performed an immunoblot analysis of TRAF2. We found robust Lys63-linked ubiquitination of both wildtype and TRAF2 S102A. In contrast, we failed to find

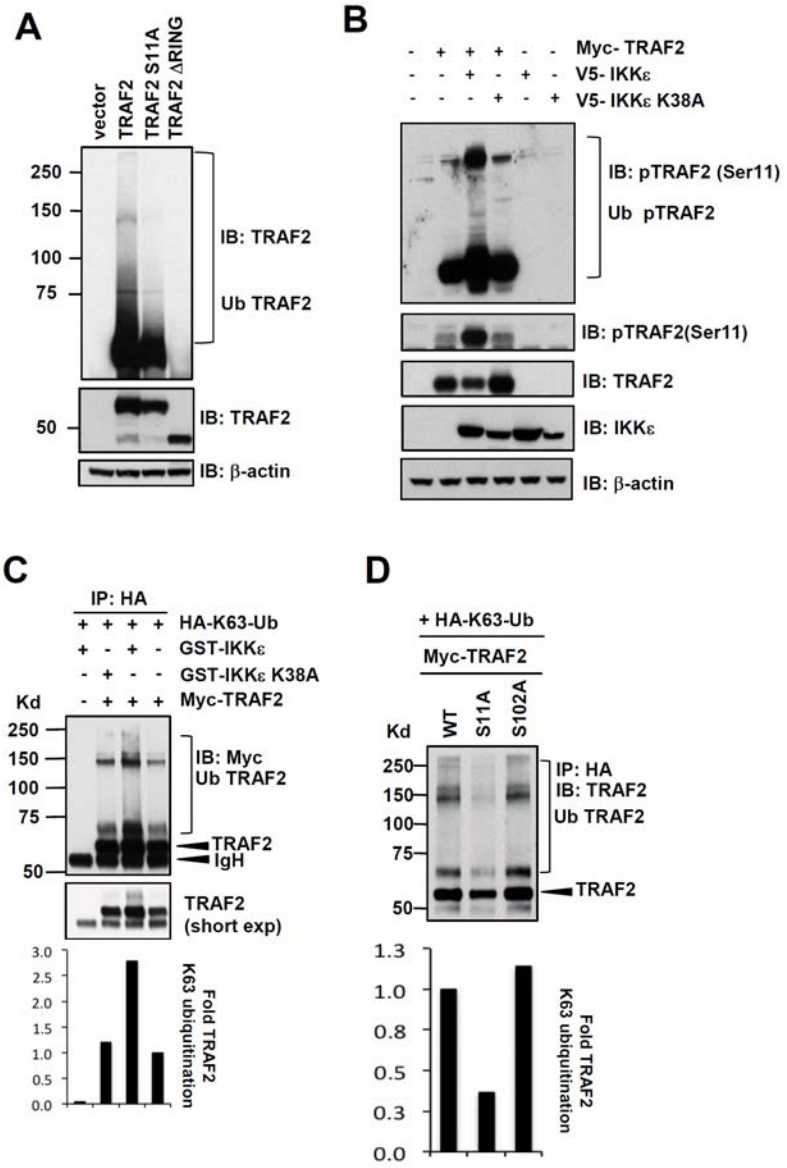

Figure 4.7. IKKε promotes Lys63-linked TRAF2 ubiquitination through phosphorylation of Ser11

(A) TRAF2 ubiquitination is dependent on Ser11 phosphorylation. HEK293T cells were transfected as indicated. Lysates were analyzed by immunoblot with antibodies specific for TRAF2 and β-actin. (B) HEK293T cells were transfected as indicated. Lysates were analyzed by immunoblot with antibodies specific for TRAF2, IKKε and pTRAF2 (Ser11). (C) Lys63-linked TRAF2 ubiquitination in the presence of IKKε. HEK293T cells were cotransfected as indicated with HA-K63-Ub. Immunoprecipitation was performed using an HA antibody followed by immunoblot analysis of Myc-TRAF2. Short exposure shows Myc-TRAF2 above IgH (D) Lys63-linked ubiquitination of wildtype TRAF2 and TRAF2 phosphorylation mutants. HA-K63-Ub immune complexes were isolated from HEK293T cells cotransfected as indicated with HA-K63-Ub and analyzed by immunoblot with a TRAF2 antibody.

evidence of Lys63-linked ubiquitination of TRAF2 S11A (Figure 4.7D). Thus, IKKε promotes TRAF2 Lys63-linked ubiquitination in a manner that is dependent on Ser11 phosphorylation.

TRAF2 is essential for IKKε-mediated NF-κB activation and cell transformation

Since IKKε phosphorylates TRAF2 to promote its Lys63-linked ubiquitination and NF-κB activation, we investigated whether *TRAF2* was essential for IKKε-induced NF-κB activation and transformation. Using two distinct *TRAF2*-specific shRNA, we suppressed *TRAF2* expression in HEK293T cells and two IKKε-transformed cell models, HA1EM and HMLEM cells that stably express IKKε. We found that *TRAF2* suppression strongly inhibited IKKε-induced NF-κB activity (Figure 4.8A) and suppressed anchorage-independent colony formation in HA1EM and HMLEM cells (Figure 4.8B, C, D and E). These results indicate that TRAF2 is required for transformation driven by IKKε.

To determine whether TRAF2 Ser11 phosphorylation is necessary for IKKε-induced transformation, we generated IKKε-transformed HA1EM cells that stably express wildtype TRAF2, TRAF2 S11A, and TRAF2 S102A (Figure 4.9A). As expected, we found that cells expressing F-IKKε showed robust anchorage-independent growth (Figure 5F). However, expression of TRAF2 S11A inhibited anchorage-independent colony growth by 42%, suggesting that this mutant acts as a dominantly interfering mutant (Figure 4.9B). These findings demonstrate that phosphorylation of TRAF2 at Ser11 is necessary for IKKε-mediated cell transformation.

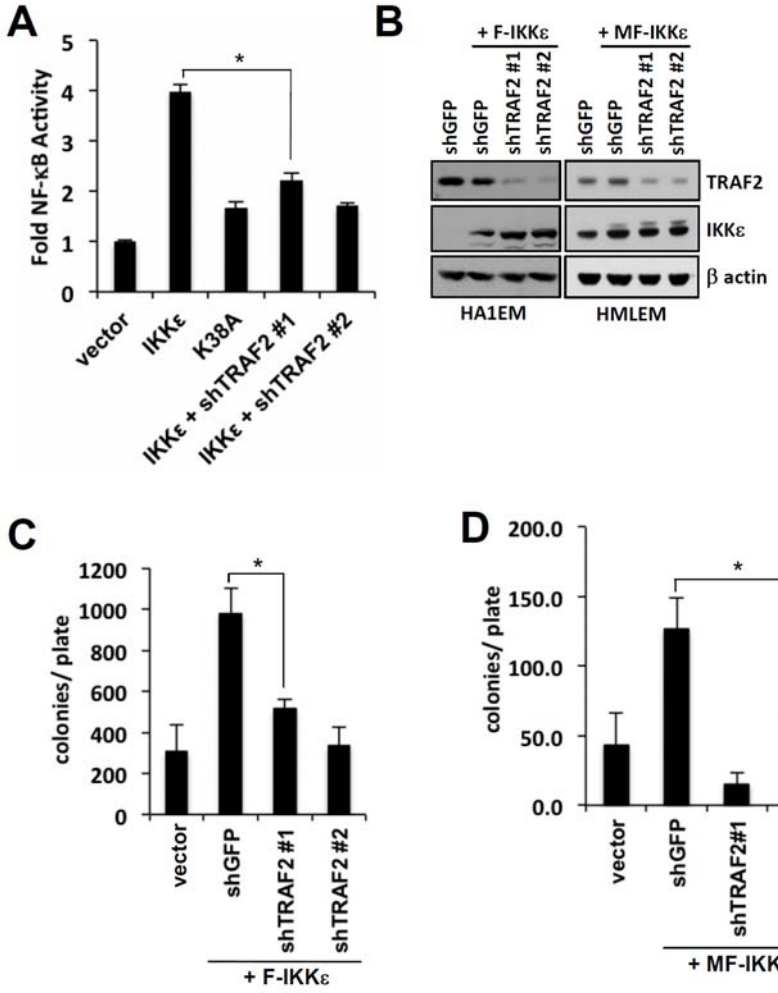

Figure 4.8. TRAF2 is required for IKKε-induced NF-κB activation and transformation

(A) IKKε -induced NF-κB activity following suppression of *TRAF2*. HEK29T cells were cotransfected as indicated with SV40-renilla and a NF-κB luciferase reporter. Raw light unit (RLU) activity was normalized to activity observed with control vector. Results reported as mean ± SD of three experiments. *p = 5.2 x 10-5, calculated by a standard t-test. (B) *TRAF2* suppression in IKKε -transformed HA1EM and HMLEM cells. Immunoblot of TRAF2 and IKKε in control IKKε-transformed HA1EM and HMLEM cells following transduction with shTRAF2#1, shTRAF2#2 or control shGFP. Results show β-actin loading control. (C) Anchorage-independent growth of IKKε -transformed HA1EM cells following *TRAF2* suppression. Colony formation of cells from (B) was analyzed after 28 d. Results reported as mean ± SD of three experiments. *p = 4.7 x 10-5, calculated by a standard t-test. (D) Anchorage-independent growth of IKKε- transformed mammary cells following *TRAF2* suppression. Colony formation of cells from (B) was analyzed after 28 d. Results reported as mean ± SD of three experiments. *p = 0.0011, calculated by a standard t-test.

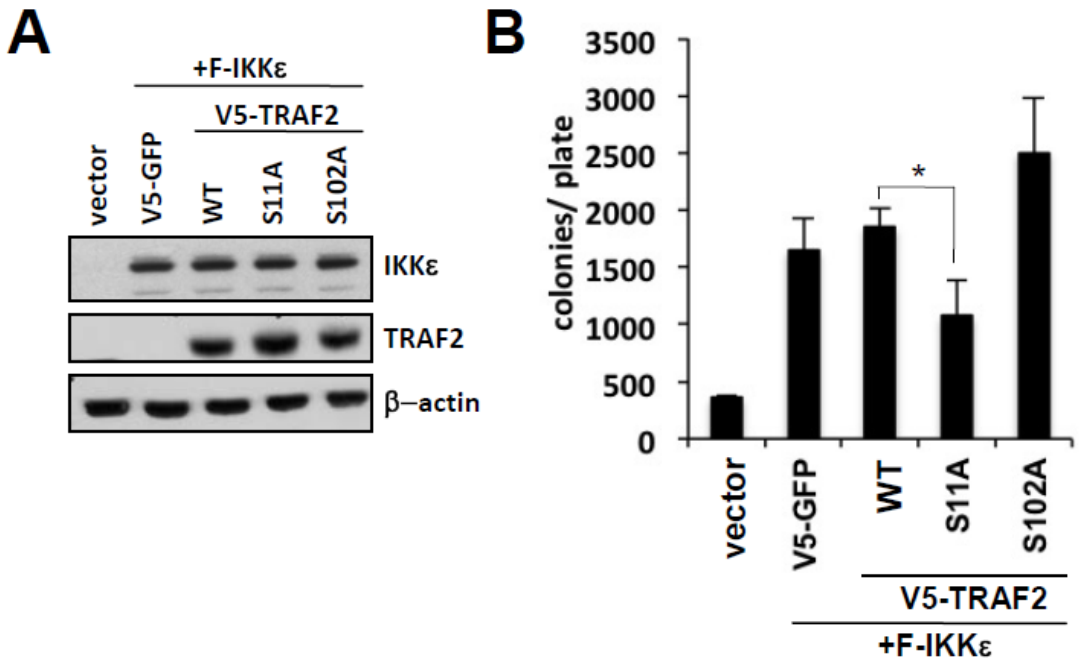

Figure 4.9. TRAF2 Ser11 phosphorylation is required for IKKε-mediated transformation

(A) TRAF2 overexpression in IKKε -transformed HA1EM cells. Immunoblot of TRAF2 and IKKε in control (vector) HA1EM or HA1EM- F-IKKε cells transduced with V5-GFP, V5-TRAF2, V5-TRAF2 S11A, or V5-TRAF2 S102A. Results show β-actin loading control. (B) Anchorage-independent growth of IKKε -transformed cells in the presence of TRAF2. Colony formation of cells from (E) were assayed after 28 d. Results reported as mean ± SD of three experiments. *p = 0.0045, calculated by a standard t-test.

IKKε-dependent breast cancer cells are also dependent on TRAF2

Since TRAF2 is an IKKε substrate necessary for IKKε-induced transformation, we sought to determine whether TRAF2 is essential in IKKε-dependent breast cancer cell lines. We first measured IKKε expression and TRAF2 Ser11 phosphorylation levels in several breast cancer cell lines. We identified five lines (BT549, MDA-MB-453, ZR-75-1, MCF-7 and T47D) with increased IKKε expression that also showed an increased level of TRAF2 Ser11 phosphorylation as compared to total TRAF2 levels (Figure 4.10A). In accordance with our prior findings, these cell lines also exhibited a decreased proliferative capacity in a long-term proliferation assay when we suppressed IKKε with two independent IKKε-specific shRNA (Figure 4.10B). When we suppressed *TRAF2* in these IKKε–dependent breast cancer cell lines, we observed a similar decrease in cell proliferation as well (Figure 4.10B). In contrast, breast cancer cell lines that do not depend on IKKε expression exhibited no change in proliferation after TRAF2 depletion. Together these observations identify TRAF2 as an IKKε substrate necessary for IKKε-induced transformation.

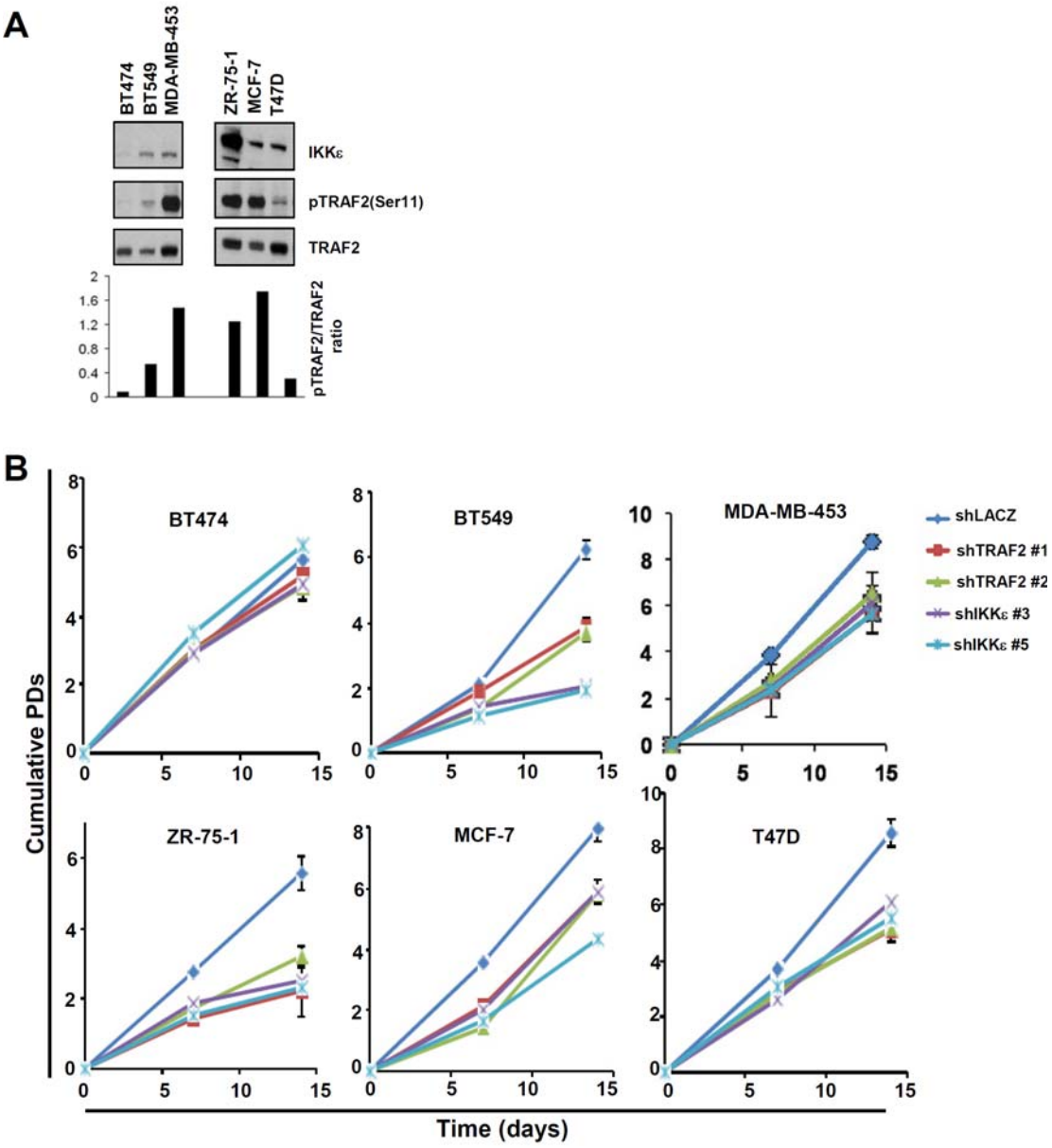

Figure 4.10. TRAF2 is required for IKKε-mediated transformation
(A) Levels of IKKε, pTRAF2 (Ser11) and total TRAF2 in breast cancer cell lines. Immunoblot was performed in the indicated breast cancer cell lines. A ratio of pTRAF(ser11)/total TRAF2 was calculated by densitometry and shown in the lower panel. (B) Long-term proliferative capacity of cell lines following IKKε and TRAF2 suppression. Population doubling (PD) of breast cancer cell lines from (A) after transduction with shLacZ, shTRAF2#1, shTRAF2#2, shIKKε#3 or shIKKε#5. Cells were assayed for 14 d. Culmulative PDs are shown on the vertical axis, Time in days is shown on the horizontal axis.

DISCUSSION

IKKε phosphorylates and regulates TRAF2

TRAF2 is an adaptor molecule that mediates a series of ubiquitination events that facilitate NF-κB activation [20] and is itself modified by ubiquitination [17]. Recent work indicates that TRAF2 is phosphorylated at various serine residues important for NF-κB activation [16, 21-23]. In particular, TRAF2 Ser11 phosphorylation has been previously reported and this activity occurs constitutively in some cancer cell lines [16]. Here, we demonstrate that IKKε phosphorylates TRAF2 both *in vitro* and *in vivo*. We performed a mutational analysis of five TRAF2 serine residues that matched the IKKε recognition motif and identified Ser11 as the residue on TRAF2 that is phosphorylated by IKKε. This activity was necessary for TRAF2 Lys63-linked ubiquitination, NF-κB activation and subsequent transformation.

Phosphorylation of TRAF2 at Ser11 is required for TRAF2 Lys63-linked ubiquitination. Phosphorylation and subsequent Lys63-linked ubiquitination of TRAF2 at serine 11 is necessary for IKKε-mediated NF-κB activation and transformation. Since CIAP1 regulates TRAF2 stability, the interaction of IKKε and CIAP1 may be involved in the regulation of TRAF2 stability. CIAP1 plays multiple roles in promoting TRAF2 ubiquitination, attenuating caspase activation and apoptosis, and promoting NF-κB activation [15, 24, 25]. Thus, dynamic regulation of TRAF2 ubiquitination through CIAP1 may also be modulated by IKKε.

IKKε regulates both TRAF2 and CYLD

We previously demonstrated that IKKε regulates the activity of CYLD, a tumor suppressor and inhibitor of NF-κB signaling [10]. The observations presented herein suggest that the phosphorylation of CYLD and TRAF2 by IKKε coordinates these NF-κB regulators to activate NF-κB signaling in the context of cell transformation. Since TRAF2 is also directly inactivated by CYLD through deubiquitination, TRAF2 appears to be a central mediator of IKKε transformation [26].

IKKε is amplified and overexpressed in 30% of breast cancers and is an essential gene in cells that harbor IKKε copy number gain [9]. We have determined that TRAF2 also plays an essential role in IKKε- driven breast cancers. The identification of these dependencies provides multiple therapeutic strategies in targeting the NF-κB pathway in breast cancer.

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CHAPTER FIVE

CONCLUSION

In this dissertation, I have investigated the regulation and effectors of the breast cancer oncogene, IKKε. IKKε is required for the survival of the 30% of breast cancers that harbor an *IKBKE* amplification, making IKKε an attractive target for cancer therapeutics. However, unlike other oncogenes and tumor suppressors such as Ras, BRAF and PIK3CA, current activating or protein function-altering mutations involving IKKε have not yet been identified [1-3]. Thus, it would follow that the amplification of IKKε itself somehow leads to its aberrant regulation in cancer, resulting in its oncogenic function.

Prior to its identification as a breast cancer oncogene, IKKε was shown to serve as activator of interferon signaling in innate immunity. In this context, IKKε is known to act in complex with TBK1 to phosphorylate IRF3 and IRF7 [4, 5]. In addition, as non-canonical IKK proteins, IKKε and TBK1 activate NF-κB signaling[4, 6, 7]. In the context of breast cancer, IKKε also activates NF-κB signaling and, interestingly, this NF-κB activity is required for IKKε oncogenic function [8]. Thus, we sought to define the oncogenic signaling pathway that resulted in the aberrant and NF-κB-dependent activation of IKKε. In my dissertation work, I have defined the role of ubiquitination in the regulation of IKKε (Chapter 2). In addition, through collaborative work, we have also uncovered two novel downstream targets of IKKε kinase function (Chapters 3 and 4) in the context of oncogenic cell transformation. This work, taken together, defines a novel oncogenic signaling network that drives aberrant NF-κB activation in the context of breast cancer.

IKKε REGULATION BY K63-LINKED UBIQUITINATION

Both degradation-dependent and degradation-independent ubiquitination are essential for the proper regulation of specific components in the NF-κB pathway [9]. Thus, we suspected that IKKε was also regulated by ubiquitination. In Chapter 2, I found that IKKε was indeed regulated by K63-linked degradation-independent ubiquitination. I further identified K30 and K401 to be the critical residues of IKKε modification and found that ubiquitination of IKKε at these two residues is required for proper IKKε oncogenic function. In addition, I identified TRAF2 as a putative E3 ligase that is responsible for IKKε K63-linked ubiquitination in the context of breast cancer.

Here, I have also identified a possible structural explanation for the essentiality of IKKε ubiquitination. Ongoing collaborative work with Daqi Tu, Michael Eck, Zehua Zhu and David Barbie (in submission) has solved the protein crystal structure of TBK1, a close family member of IKKε with whom IKKε shares ~65% similarity. This work has found that TBK1 homodimerization is required for TBK1 kinase function, downstream IRF3 phosphorylation and NF-κB activation. Residues K30 and K401 are conserved between IKKε and TBK1, and I have found that, like IKKε, TBK1 is also ubiquitinated on K30 and K401. In the TBK1 protein structure, the conserved K30 and K401 residues are found on opposing faces of the dormant monomer but become juxtaposed on an exposed face when the catalytically-active dimer is formed. Thus, this data suggests that K63-linked ubiquitination of IKKε and TBK1 at these two critical residues is important for the interface of the

kinase with other regulatory proteins and binding partners. In future studies, it would be interesting and informative to determine which proteins interact with this interface, and how these interactions are regulated both in the context of cancer and innate immunity.

Importantly, my work has demonstrated that the K30 and K401 ubiquitination defective IKKε mutants are still able to bind to the downstream substrate, CYLD. This observation makes it unlikely that these mutants disrupt the overall structure of IKKε. Instead, these data suggest that ubiquitination at K30 and K401 induces an allosteric change to regulate kinase activity, or that these mutations affect the recruitment of other molecules critical for IKKε kinase function. Thus further studies are needed to determine the mechanism by which IKKε ubiquitination regulates its kinase function.

In my dissertation work, I have also defined a novel role for TRAF2 as an E3 ligase that modifies IKKε with K63-linked ubiquitin chains. TRAF2 is known to interact in the same complex with TBK1, cIAP1, and TANK [4, 10]. Previously, TRAF2 has been characterized as the E3 ligase that is responsible for RIP1 K63-linked ubiquitination in the context of the canonical NF-κB pathway [11]. It has also been shown that cIAP1 and cIAP2 are capable of catalyzing RIP1 ubiquitination as well [12]. Here, I have demonstrated that TRAF2, but not TRAF6, is required for IKKε ubiquitination and that its RING domain is essential for its function. However, I have not extensively surveyed the entire collection of E3 ligases that are capable of catalyzing K63-linked ubiquitination. In addition, although the mass spectrometry analysis has covered the majority of the internal lysines within IKKε (22 out of 34,

64.7%) there still remain a set of residues that have not been surveyed. Although I have identified two essential residues for IKKε ubiquitination and subsequent downstream function, K30 and K401, it is certainly possible that there are more modified residues that have yet to be studied. Polyubiquitination is a very dynamic and often transient form of regulation. The analysis here has identified one particular mechanism of IKKε regulation by ubiquitination, but more comprehensive work in the future will be required in order to define all the residues, ubiquitin linkage types, and E3 ligase enzymes that play a role in this complex process.

IDENTIFYING THE DOWNSTREAM TARGETS OF IKKε ONCOGENIC ACTIVATION

In collaborative work, we have used an unbiased peptide screen to define the downstream targets of IKKε kinase function [13, 14]. Through the use of this screen, we have identified two substrates, TRAF2 and CYLD, as effectors of IKKε oncogenic signaling.

In Chapter 3, we identified CYLD Ser418 to be a direct phosphorylation target of IKKε. CYLD is already known to be tumor suppressor whose mutation leads to the development of familial cylindromatosis, an autosomal dominant disease characterized by the formation of benign skin tumors [15]. We found IKKε phosphorylation of CYLD at Ser418 to be an inhibitory modification, resulting in CYLD loss of function. We determined that CYLD Ser418 phosphorylation is essential for IKKε-mediated transformation and CYLD deubiquitinase function.

In Chapter 4, we also identified TRAF2 Ser11 to be a direct phosphorylation target of IKKε. Phosphorylation of TRAF2 at Ser11 results in its activation by K63-linked ubiquitination. We further determined that TRAF2 phosphorylation at Ser11 is essential for IKKε-mediated transformation and subsequent NF-κB activation.

CYLD and TRAF2 have been shown to be essential regulators of normal NF-κB signaling. CYLD is known to associate with and deubiquitinate NEMO, the regulatory subunit of the IKK complex, thereby negatively regulating canonical NF-κB activation [16, 17]. TRAF2 has been well-studied to activate the canonical NF-κB pathway by mediating the signaling between the proinflammatory cytokine receptors and the TAK complex, an activator of the canonical IKK complex [9, 18]. Together, the activation of TRAF2 and inactivation of CYLD results in the cooperative upregulation of canonical NF-κB signaling. Previous work has defined IKKε-mediated oncogenic transformation to also be dependent on canonical NF-κB signaling. Specifically, the introduction of a non-degradable form of IκBα, known to be a super-repressor of canonical NF-κB signaling, resulted in the ablation of IKKε-mediated transformation as assessed by soft agar colony formation [8]. Though this data was quite striking, it remained unclear why a super-repressor of the canonical NF-κB pathway would have an effect on the signaling of a non-canonical IKK protein. In this dissertation work, we have demonstrated that, in the context of cancer, IKKε directly activates TRAF2 and inactivates CYLD, two effectors in the canonical NF-κB pathway. These data not only offer a mechanistic explanation for how IKKε mediates transformation but, further answer the question about why IKKε-mediated transformation is dependent on the canonical NF-κB pathway.

Importantly, although these studies have shown TRAF2 and CYLD to be major contributors towards IKKε-mediated transformation, there are several other putative IKKε substrates that were identified in our unbiased peptide screen that may also be IKKε effectors. These other substrates may prove not only to be important players in IKKε-mediated oncogenic function, but also effectors of IKKε function in innate immunity and NF-κB activation. For future studies, identification and characterization of these putative IKKε substrates may provide further insight into the mechanism of IKKε function both the context of oncogenic and normal signaling.

DEFINING A FEED-FORWARD NETWORK OF IKKε REGULATION

The intersection of the work presented in this dissertation defines an interesting feed-forward network of IKKε regulation, illustrated in Figure 5.1. I have shown that IKKε is positively regulated by TRAF2, a known K63-linked E3 ligase [10, 11]. In addition, we have shown that TRAF2 itself is also a downstream target of IKKε phosphorylation. We have also shown that IKKε phosphorylates CYLD, a deubiquitinating enzyme (DUB) that removes Lys63 linked ubiquitin chains and acts as a negative regulator of NF-κB signaling [16, 17, 19]. Interestingly, one direct substrate of CYLD deubiquitination is TRAF2. Thus, it would seem that we have defined a feed-forward loop in which IKKε activation results in further activation of TRAF2, leading to even more IKKε activation. This mode of regulation may explain why IKKε amplification is such a potent driver as a breast cancer oncogene.

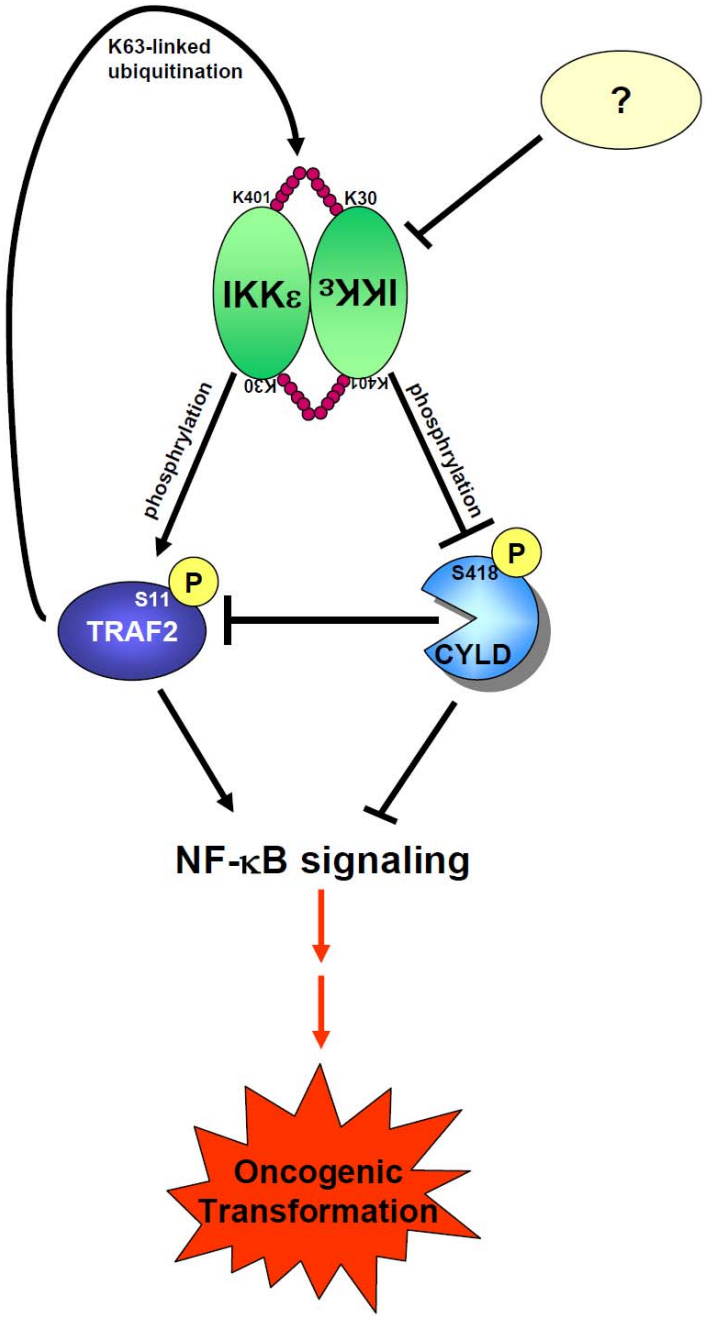

Figure 5.1. The IKKε oncogenic network in cancer.
IKKε, in dimer form is positively regulated by TRAF2-mediated K63-linked ubiquitination at K30 and K401. Activated IKKε phosphorylates and activates TRAF2 at S11 and inactivates CYLD at S418, resulting in the feed forward activation of IKKε, activation of aberrant NF-κB signaling, and eventual oncogenic transformation.

However, as we know that IKKε is physiologically activated and expressed in innate immunity, it is likely that we still do not have a full understanding of how IKKε operates in the response to viral infections. In the context of innate immunity, IKKε is tightly regulated in a way that allows it to be turned on and off in the presence or absence of viral infection. Thus, there must exist a mode of regulation that allows for IKKε signaling to be turned off. This could exist in the form of a deubiquitinating enzyme that removes IKKε K63-linked ubiquitination and thereby inhibits it. It is also possible that IKKε is negatively regulated by a different kind of modification, perhaps an inhibitory phosphorylation or degradation-dependent ubiquitination. Alternatively, IKKε could also be negatively regulated by a binding protein that either sequesters IKKε protein to a separate cell compartment or binds to IKKε in such a way that inhibits IKKε K30 and K401 ubiquitination. More studies are required to determine how IKKε is negatively regulated and to more completely define the network of IKKε regulation in oncogenic transformation.

In addition, all of these studies were performed only in the context of IKKε amplification and overexpression in breast cancer. It is likely that the overexpression of IKKε results in its dysregulation, allowing it to take on novel functions that are usually not active in the normal cell. Thus, the model of specific effectors and modes of regulation that we have defined here may not hold true in the context of normal IKKε function. Therefore, more studies are required to determine if this model of IKKε regulation and downstream signaling also occurs in the context of interferon regulation.

IKKε AS A THERAPEUTIC TARGET

IKKε has been shown to be a specific vulnerability for the ~30% of breast cancers that harbor an *IKBKE* amplification or IKKε overexpression. This has made IKKε an attractive candidate as a therapeutic target. However, the significant kinase domain homology between IKKε and TBK1 makes it unlikely that ATP-competitive small molecule inhibitors that inhibit IKKε but not TBK1 will be found. Thus, the work in this dissertation presents alternative ways to inhibit IKKε function.

Recent work has demonstrated that E3 ligases can also be targeted and inhibited by small molecule inhibitors [20]. In particular, small molecule inhibitors of the cullin-RING family of E3 ubiquitin ligases have been described [21, 22]. We found that TRAF2-mediated K63-linked ubiquitination is essential for IKKε kinase activity and also that TRAF2 is a downstream target of IKKε that functions as an IKKε oncogenic effector. Thus these observations may identify an alternative mechanism to ultimately target IKKε therapeutically in the context of human disease.

Taken together, the work that has been presented in this dissertation has elucidated a novel regulatory pathway for IKKε in the context of breast cancer. We have identified key molecular players in the regulation of IKKε as well as important downstream effectors of IKKε function. Though further studies are required to complete the picture, the work presented in this dissertation has defined the first components of a novel oncogenic network for the breast cancer oncogene and NF-κB effector, IKKε.

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